REMARKS

By this amendment, Applicants have amended claims 70, 82, 83, 88, 89, 91, 92, and 97 and have amended the specification to correct minor typographical errors. No new matter has been added. Applicants respectfully request entry of the amendment and allowance of the pending claims.

In the Office Action, the Examiner requested Applicants to supply the four non-patent references appearing on the second page of the information disclosure statement filed March 5, 2002. The requested non-patent references are transmitted herewith. Applicants respectfully request that the Examiner initial the information disclosure statement to show that the enclosed references were considered.

Rejections Under 35 U.S.C. § 112, First Paragraph

The Examiner rejected claims 70 and 82-97 under 35 U.S.C. §112, first paragraph, as allegedly containing new subject matter. The Examiner alleged that the formula of claim 70 lacks a point of attachment for the L' and NUC as described in the specification. The Examiner further alleged that the subject matter of claims 82-97 is not supported by the specification.

Applicants respectfully disagree with the Examiner that claims 70 and 82-97 contain new matter. However, to expedite prosecution, Applicants have amended claim 70 to further clarify the L' and NUC terms. Support for the amendment can be found, for example, on pages 77, lines 18-24 and on page 76, lines 9-24. No new matter has been added.

With regard to claims 82-97, support for these claims can be found throughout the specification and claims as originally filed. For example, support for claim 82 can be found on page 33 formula I, on page 40 as formula Ib, on page 43 as formula P.1, and on page 74 as formula IIIb. Support for claim 83 can be found, for example, on pages 45-46 in formulas (Y-1) to (Y-4), and in claim 19 as filed. Support for claim 84 can be found, for example, on pages 47, line 23 to page 48, line 9. Support for claims 85 and 86 can be found, for example, on page 35, lines 14-22. Support for claim 87 can be found, for

example, on page 52, lines 27-28. Support for claim 88 can be found, for example, on page 76, lines 25-30. Support for claim 89 can be found, for example, on page 77, line 3. Support for claim 90 can be found, for example, on page 43, lines 7-16. Support for claim 91 can be found, for example, on pages 50 (formula Y-41a), and pages 53-56 (formulas Y-20b to Y-46b). Support for claim 92, can be found, for example, on page 57, formula Ic, on page 74, formula IIIb, and page 71, line 25, which indicates that R^{41} can be –C(O)—R^{42}, wherein R^{42} can be NH. Support for claim 93 can be found in claim 88 as filed. Support for claim 94-96 can be found, for example, on page 67, lines 10-20 and on page 68, lines 7-13. Support for claim 97 can be found, for example, on page 75, formulas IVa and IVb. Accordingly, Applicants respectfully submit that the claims are supported by the specification and request withdrawal of the rejections.

Rejections Under 35 U.S.C. § 112, Second Paragraph

The Examiner rejected claims 71-81 and 98-105 under 35 U.S.C. §112, second paragraph, for allegedly being indefinite for reciting the term "NUC". The Examiner alleged it was unclear whether NUC is equivalent to the formula of claim 70, or is equivalent to B in claim 70.

Applicants respectfully traverse this rejection and submit that the meaning of "NUC" is clear to a person of ordinary skill in the art upon reading the claims. Nevertheless, to expedite prosecution, Applicants have amended claim 70 to further define the term "NUC". Applicants submit that the amendment to claim 70 moots the Examiner's rejections under 35 U.S.C. § 112, second paragraph, with respect to claims 71-81 and 98-105. Accordingly, Applicants submit that claims 71-81 and 98-105 are in condition for allowance and request withdrawal of the rejections.

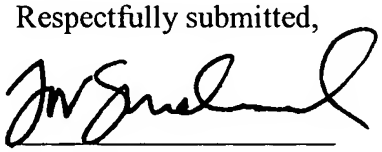
Conclusion

In view of the foregoing amendments, and the remarks set forth above, reconsideration and allowance are respectfully requested.

Enclosed is the fee for a three-month extension of time. No additional fee is believed to be due with respect to the filing of this amendment. If any additional fees are due, or an overpayment has been made, please charge, or credit, our Deposit Account No. 11-0171 for such sum.

If the Examiner has any questions regarding the present application, the Examiner is cordially invited to contact Applicant's attorney at the telephone number provided below.

Respectfully submitted,



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Novel Derivatization of Protein Thiols With Fluorinated Fluoresceins

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Abstract: Fluorescein diacetate analogs 3a-3c are shown to react selectively with protein sulfhydryl groups at neutral pH in aqueous solution, by means of substitution at the perfluorinated ring. The acetate moieties are cleaved by treatment with aqueous carbonate or by endogenous esterases, giving a fluorescent protein conjugate. Copyright © 1996 Elsevier Science Ltd

Traditional derivatization of free sulfhydryl groups in proteins with fluorescent reagents involves reaction with fluorochromes containing haloacetamides, maleimides, aziridines, halomethylaryl groups, and pyridyl disulfides.[1] Of these, fluorescent iodoacetamides are used most frequently because of the speed of reaction at neutral pH.[2] However, iodoacetamides suffer from instability to light, particularly in solution. Also, iodoacetamides can be susceptible to oxidation in DMSO, a widely used co-solvent for the preparation of fluorochrome-protein conjugates. Additionally, iodoacetamides are often difficult to prepare in pure form, sometimes necessitating the use of chloroacetamides as intermediates.[3] Thus, there is still a substantial need for improved fluorescein, sulfhydryl-reactive probes.[4] Herein we report a novel means of derivatizing protein thiol groups involving nucleophilic aromatic substitution of fluorinated fluoresceins.

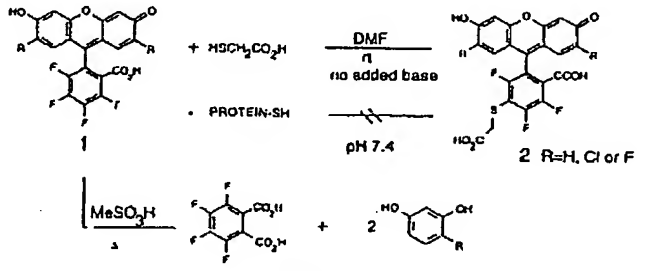

Figure 1

Nucleophilic displacement of activated aromatic fluorines is well known. A critical requirement for facile reaction is the presence of an electron-withdrawing group *ortho* or *para* to the fluorine atoms.[5] Reaction of the fluorinated fluorescein **1**[6] with thiols such as mercaptoacetic acid in organic solvents was facile *in the absence of added base*.[7] The reaction was highly selective for substitution at the 6-position, *para* to the carboxylic acid (Figure 1). However, at near neutral pH in water, no reaction was observed between **1** and *E. coli* β-galactosidase, a thiol-containing enzyme. We rationalized this result by assuming that the carboxylic acid is mostly deprotonated at neutral pH in aqueous solution, thus deactivating the tetrafluorinated ring of **1** toward substitution. In organic solution the carboxylate is protonated and thus electron-withdrawing, allowing for facile *para*-substitution (Figure 1).

Acetylation of the phenolic hydroxyl groups is a way to stabilize the electron-withdrawing character of the carboxylate in aqueous solution, by forcing the dye into a nonfluorescent spirolactone form **3** (Figure

2).[8] Gratifyingly, reaction of β-galactosidase with 3a at near neutral pH readily resulted in protein modification (Figure 2). Simply raising the pH briefly to 10 cleaved the acetates from the dye, generating the fluorescently labeled protein 5a.[9] Purification by gel-filtration chromatography readily separated the protein conjugate from free dye, and allowed reaction stoichiometry to be determined.

High selectivity for sulfhydryl groups is evidenced by the lack of reaction between 3a and IgG (contains only cystine residues), and between 3a and streptavidin (contains no cysteine residues), under identical conditions. In contrast, pretreatment of IgG with dithiothreitol (DTT) to liberate free sulfhydryl groups gave the expected reaction product (5a) with 3a.

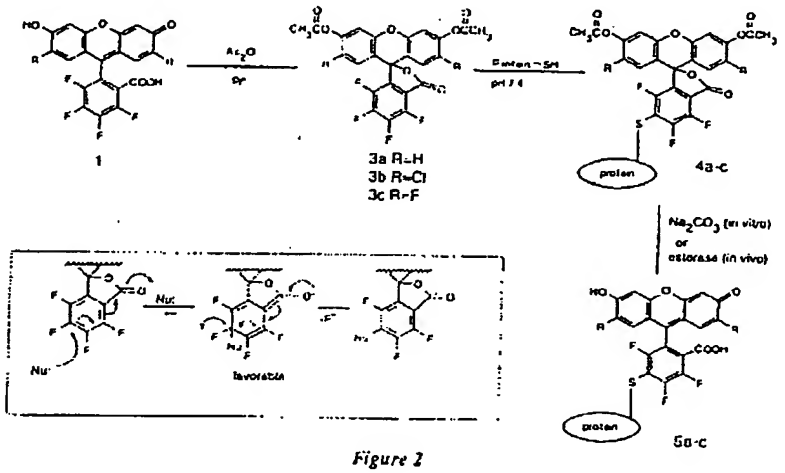

Figure 2

We also prepared the 2′,7′-dichloro (3b, R=Cl) and 2′,7′-difluoro (3c, R=F) analogs. Table 1 shows the relative reactivity with β-galactosidase of all three probes, as measured by the degree of substitution, from a representative set of experiments.[10] In addition to reaction with proteins *in vitro*, diacetates 3 were also shown to react with biomolecules in intact cells. Cultured human lymphoid B-cells were incubated with 1 μM solutions of 3, followed by washing with fresh medium.[11] For quantitation of dye binding to cellular macromolecules, the cells were fixed by formaldehyde treatment and analyzed by flow cytometry (Table 1). In this case it is probable that endogenous esterases cleave the acetates from the fluorochrome, obviating the basic hydrolysis step. To confirm that the dyes were labeling cellular proteins, the labeled cells were lysed, and the lysate was analysed by gel electrophoresis,[12] which showed that a number of fluorescently labeled protein bands had been produced (data not shown). Qualitatively, the bands from cells stained with 3a appeared brighter than those stained with 3b and 3c, consistent with the fluorescence intensity values determined by flow cytometry and *in vitro* protein labeling results.

In conclusion, we have developed fluorinated fluorescein diacetates (3) that readily and selectively react with protein sulfhydryl groups in a novel protein conjugation method. The ease of synthesis and purification of 3 makes these reagents more attractive than traditionally used thiol-reactive fluorescent probes. These reagents add a new means for selective protein derivatization to the repertoire of the bioconjugate chemist.

Table 1. Labelling of Proteins in Vitro and in Cells with 3a-3c

	DOS[a]	DOS[b]	Signal[c]
Dye 3a	5.0	8.6	2450±88
Dye 3b	2.3	4.4	1470±66
Dye 3c	2.8	3.9	400±26
Control (no dye)			2±1

DOS = Degree of substitution.
a 10:1 Dye:protein molar ratio b 20:1 Dye:protein molar ratio
c Relative fluorescence emission intensities of cell samples stained in parallel with 1 μM dye, measured by flow cytometry[11]; n=4

References & Notes

1. Lundblad, R.L.; Noyes, C.M., *Chemical Reagents for Protein Modification*, Vol. I, CRC Press, New York, 1984.
2. Brinkley, M. *Bioconjugate Chemistry* 1992, 3, 2-13.
3. Corrie, J.E.T; Craik, J.S. *J. Chem. Soc. Perkin Trans. I* 1994, 2967-2973.
4. For examples of recent efforts, see: a) Langmuir, M.E.; Yang, J.-R.; Moussa, A.M.; Laura, R.; LeCompte, K.A. *Tetrahedron Letters* 1995, 36, 2989-2992; b) Walker, M.A. *J. Org. Chem.* 1995, 60, 5352-5355; c) Corrie, J.E.T. *J. Chem. Soc. Perkin Trans. I* 1994, 2975-2982.
5. Bolton, R.; Sandall, J.P.B. *J. Chem. Soc. Perkin Trans. II* 1978, 1288-1292, and references cited therein.
6. For example, tetrafluorophthalic acid (3.72g, 15.6 mmol) and resorcinol (3.43 g, 31.2 mmol) were heated in methanesulfonic acid (50 ml) for 48 hours at 80-90°C. The reaction solution was poured into 400 ml water. After cooling, the precipitate was filtered, rinsed with water (100 ml), and dried *in vacuo* to give 3.68 (91%) of a brick-red powder: ^{1}H NMR (d_6-DMSO) δ 7.00 (d, J=8.7, 2H), 6.70 (d, J=2.3, 2H), 6.60 (dd, J=8.7, 2.3, 2H); ^{19}F NMR (d_6-DMSO) Φ 135.5 (m, 1F), 139.6 (m, 2F), 147.5 (t, 1F); ε 85, 600 cm^{-1}M^{-1} (508 nm, pH 9). Anal. calcd for $C_{20}H_8O_5F_4$: C, 59.42; H, 1.99. Found: C, 59.39; H, 2.01. Resorcinol and 4-chlororesorcinol are commercially available. For a synthesis of 4-fluororesorcinol, see Patrick, B.T.; Darling, D.L. *J. Org. Chem.* 1986, 51, 3242-3244.
7. For example, a solution of 1 (R=F, 0.75 g, 1.70 mmol) and mercaptoacetic acid (0.13 mL, 1.80 mmol) in DMF (9 mL) is stirred for 24 hr at rt, then poured into 100 mL cold water. The resulting precipitate is collected, rinsed with water, and dried *in vacuo* to give 2 (R=F) as 0.86 g (99%) of an orange powder: ^{1}H NMR (d_6-DMSO) δ 7.05 (t, J=11.1 Hz, 2H), 6.88 (dd, J=7.5, 2.8 Hz, 2H), 3.84 (s, 2H). ^{19}F NMR (d_6-DMSO) Φ 110.65 (br s, 1F), 120.62 (d, 1H), 134.84 (br s, 2F), 138.15 (br s, 1F). Anal. calcd for $C_{22}H_6F_4O_5S{\cdot}H_2O$: C, 49.82; H, 2.09. Found: C, 49.56; H, 2.32. The reaction proceeded more slowly in acetonitrile.
8. For example, 1 (R=H, 45.1 g, 0.111 mol) is heated briefly with ten equivalents of acetic anhydride in pyridine (100 mL). After cooling, water (300 mL) is added to precipitate 3a (R=H), which is collected and crystallized from ethyl acetate/hexanes to give the pure product as 43.1 g (80%) of a colorless powder: ^{1}H NMR ($CDCl_3$) δ 7.15 (d, J=22 Hz, 2H), 6.99 (d, J=8.7Hz, 2H), 6.92 (dd, J=8.7, 2.2 Hz, 2H), 2.31 (s, 6H); ^{19}F NMR ($CDCl_3$) Φ 137.26 (m, 1H), 140.79 (m, 1H), 141.21 (m, 1F), 148.98 (m, 1F). Anal. calcd for $C_{24}H_{12}F_4O_7$: C, 59.03;

H, 2.48. Found. C, 59.00; H, 2.69.

9. In a typical coupling experiment, *E. coli* β-galactosidase (Boehringer Mannheim, EC 3.2.1.23, 3 mg) was dissolved in 150 μL 0.1 M phosphate buffered saline (PBS) at pH 7.5. To this was added 13.6 μL of a stock solution of 3a (5 mg in 500 μL DMSO), giving a molar ratio of 20:1 dye:protein. After 1 hr, TLC showed both 4a and 3a, and the pH was raised by addition of 200 μL of 1 M Na_2CO_3 to pH 10. After another 2 hr, the unreacted dye was removed from the conjugate using a P-30 spin column and pH 7.5 PBS. The conjugate contained no free dye, as judged by TLC analysis. The absorbance maxima of the conjugated dye (515 nm) and native protein (280 nm) were measured at pH 7.5. The absorbance at 280 nm was corrected for the absorbance of the free dye (1) at 280 nm and the degree of substitution (DOS) calculated using an extinction coefficient of 85,600 $cm^{-1}M^{-1}$ at 515 nm for the free dye (1a).
10. *E. coli* β-galactosidase contains 64 cysteine residues/enzyme, (Craven, G.R.; Steers, E.; Anfinsen, C.B. *J. Biol. Chem.* 1965, 240, 2468-2477) with varying amounts oxidized as cystine bridges, depending on preparation and purification methods. According to the manufacturer, the lots used for the experiments described herein were determined to contain 16.0 free sulfhydryl groups/molecule. Titration with thiol reagents has demonstrated that modification of a large number of cysteinyl residues occurs without loss of enzyme activity (Wallenfels, K.; Müller-Hill, B : Dabich, D.; Streffer, C.; Weil, R. *Biochem. Z.* 1964, 340, 41-55).
11. Human B-cells were cultured in RPMI 1640 medium supplemented with 10% PBS, 100 U penicillin, 100 μg streptomycin and 300 μg L-glutamine per mL of culture medium. Stock solutions of 3 in DMSO were added to cell culture medium to obtain a final dye concentration of 1 μM. The cells were incubated at 37 °C for 30 min, centrifuged, then the staining solution was discarded. The pellets were gently resuspended in PBS and incubated for 15 min, then centrifuged again and resuspended in either fresh PBS solution (for cells to be lysed) or in warm 3.7% formaldehyde in PBS for fixation. The cells in formaldehyde were incubated twice for 10 min, centrifuged, and resuspended in fresh culture medium for flow cytometry using a FACS™-Vantage instrument equipped with an argon-ion laser (488 nm excitation); fluorescence emission was collected using a 515 nm longpass filter and a single photomultiplier tube. The sheath fluid was 0.9% NaCl, and typical sample flow rates were between 200 and 400 particles per second.
12. For gel electrophoretic analysis, labeled cells in PBS were heated briefly with SDS gel loading buffer at 90 °C, then allowed to cool and loaded onto 15% (37.5:1) polyacrylamide, 0.05% SDS gels. After electrophoresis, gels were placed directly on a UV transilluminator (300 nm) and photographed. Subsequently, the gels were stained with SYPRO® Orange, a fluorescent protein gel stain, to confirm that the bands labelled with 3 were indeed high molecular weight components and not small molecule thiols such as glutathione (Steinberg, T.H.; Jones, L.J.; Haugland, R.P.; Singer, V.L. *Anal. Biochem.* 1996, 239, 223-237; Steinberg, T.H.; Haugland, R.P.; Singer, V.L. *Anal. Biochem.* 1996, 239, 238-245).

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Nucleophilic Reactions of Pyridines and Imidazoles with Vinyl and Aromatic Halides

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Received September 11, 1992

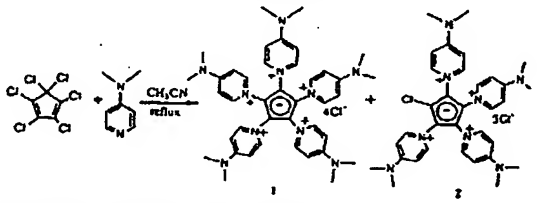
The reaction of 4-(dimethylamino)pyridine (DMAP) with hexachlorocyclopentadiene gives two products, 1,2,3,4,5-pentakis(4-(dimethylamino)pyridinium-1-yl)cyclopentadienyl anion (1) and 1-chloro-2,3,4,5-tetrakis(4-(dimethylamino)pyridinium-1-yl)cyclopentadienyl anion (2). The pK_a of the conjugate acid of 1, with protonation occurring on the cyclopentadienyl ring, is approximately −6. The reaction of DMAP with hexafluorobenzene gives 1,2,4,5-tetrafluoro-3,6-bis(4-(dimethylamino)-pyridinium-1-yl)benzene (3); its X-ray single crystal structure is presented. Upon further treatment of 3 with DMAP, 1-fluoro-2,3,4,5,6-pentakis(4-(dimethylamino)pyridinium-1-yl)benzene (4) is produced. The nucleophiles DMAP, 4-phenylpyridine, 1-methylimidazole, and 1-phenylimidazole were used with 1,2-dichlorotetrafluorocyclobutene to produce, respectively, 1,2-bis(4-(dimethylamino)-pyridinium-1-yl)tetrafluorocyclobutene (5), 1,2-bis(4-phenylpyridinium-1-yl)tetrafluorocyclobutene (6), 1,2-bis(3-methylimidazolium-1-yl)tetrafluorocyclobutene (7), and 1,2-bis(3-phenylimidazolium-1-yl)tetrafluorocyclobutene (8). The electrochemistry of some of these compounds is described.

Several years ago we reported the preparation of the first perpyridinium substituted compounds from the reaction of tetrachlorocyclopropene and 4-(dimethylamino)pyridine (DMAP): 1,2,3,3-tetrakis(4-dimethylamino)-pyridinium-1-yl)cyclopropene and 1,1,2,3,3-pentakis(4-(dimethylamino)pyridinium-1-yl)allylide.[1] The latter was found to form a room-temperature, air-stable allylide radical with a variety of oxidizing agents.[2] This discovery led us to explore related charged systems that could potentially form the basis of novel polymers, for use as modified electrode coatings and as semiconductors. Since the initial report, we have prepared and characterized several types of pyridinium- and imidazolium-substituted compounds derived from perhalogenated unsaturated compounds. Electrochemical experiments were done on the multiply charged products to determine whether they undergo reversible oxidation/reduction. In this paper we report the reactions of some pyridines and imidazoles with hexafluorobenzene, hexachlorocyclopentadiene, and 1,2-dichloro-3,3,4,4-tetrafluorocyclobutene.

Results and Discussion

Hexachlorocyclopentadiene. When hexachlorocyclopentadiene and excess DMAP were heated at reflux in acetonitrile for 4 days, 25% of 1 and 12% of 2 were obtained after recrystallization (Scheme I). Assignments are based on the following data. The ^{1}H NMR spectrum of 1 shows only one type of pyridinium and its ^{13}C NMR spectrum shows one type of pyridinium and one other signal in the aromatic region at 112.6 ppm. UV–vis spectroscopy shows λ_{max} = 308.4 nm (ϵ = 83 600) in water, and the elemental analysis was consistent with the assigned structure, assuming 4.5 waters of crystallization. The ^{1}H NMR spectrum of 2 showed two types of pyridinium, while its ^{13}C NMR spectrum showed two types of pyridinium and three other weak signals in the aromatic region. Compound 2 has λ_{max} at 305.6 nm (ϵ = 58 600) in water. The elemental

Scheme I. Reaction of Hexachlorocyclopentadiene with DMAP



1 2

analysis is consistent with the structure, assuming four molecules of water. Reaction of hexachlorocyclopentadiene with excess DMAP at 120 °C in adiponitrile gave a higher yield of 1, about 80%, in the crude reaction mixture, but the impurities were difficult to remove and reduced the effectiveness of these conditions. It should be noted that formation of 1 and 2 requires a reduction of the cyclopentadiene at some stage, perhaps via nucleophilic attack by DMAP on ring-bound chlorine.

The ^{1}H NMR spectrum of the sulfate salt of 1 in 16 M D_2SO_4 showed the α-protons of the DMAP units splitting to three signals in a 1:2:2 ratio. The β-protons split into a doublet of doublets and the dimethylamino methyl protons split into three overlapping signals. The pronounced change of the α-protons indicates that protonation occurs on the cyclopentadienyl ring. Compound 1 was completely unprotonated in 10 M DCl, and a 1:1 ratio of the protonated and nonprotonated species, as determined by integration of the α-protons of the DMAP units, was observed in 12.5 M D_2SO_4. This medium has H_0 of about −6.4,[3] hence the pK_a of the protonated 1 is about −6. This pK_a value falls between those of pentakis(trifluoromethyl)-cyclopentadiene[4] and pentacyanocyclopentadiene,[5] pK_a = −2 and −11, respectively. Both 1 and 2 are thermally stable in both the solid state and in solution, even in the presence of base, but 1 decomposed in concd D_2SO_4 over several days.

Hexafluorobenzene. Reaction of hexafluorobenzene with 5 equiv of DMAP in refluxing THF gave a yellow

(1) Waterman, K. C.; Streitwieser, A. *J. Am. Chem. Soc.* 1984, *106*, 3874.
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0022-3263/93/1958-1409$04.00/0

Scheme II. Reactions of Hexafluorobenzene with DMAP

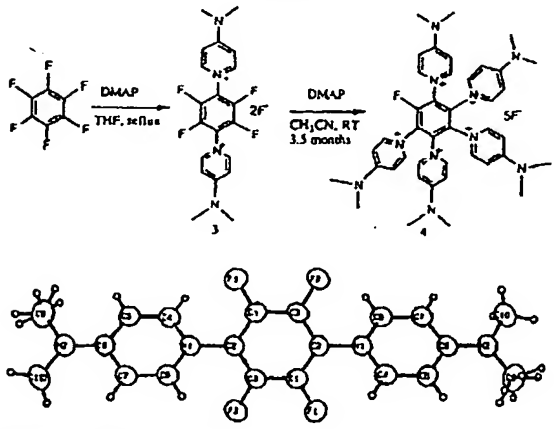

Figure 1. ORTEP drawing of 3.

Table I. Selected Bond Distances of 3

atom 1	atom 2	distance, Å	atom 1	atom 2	distance, Å
C1	F1	1.342 (2)	C1	C2	1.373 (2)
C2	N1	1.428 (2)	N1	C4	1.362 (2)
C4	C5	1.344 (2)	C5	C6	1.421 (2)
C6	N2	1.328 (2)	N2	C9	1.441 (2)

precipitate in 20% yield whose ^{1}H NMR spectrum was indicative of 1,2,4,5-tetrafluoro-3,6-bis(4-(dimethylamino)-pyridinium-1-yl)benzene difluoride (3) (Scheme II). When the reaction was allowed to stir under reflux longer than 1 day, significant amounts of other higher substituted products were formed that complicated the reprecipitations and thus lowered the isolated yield of 3. The ^{1}H NMR spectrum of 3 shows only one type of DMAP, and its ^{19}F NMR spectrum indicates two types of fluorines. Upon ion exchanging to the chloride ion, the ^{19}F NMR signal at −146 ppm disappeared, showing only one peak at −165 ppm for the covalently bound fluorine. It should be noted that the chemical shift of the fluoride ion appears to be concentration dependent. The ^{13}C NMR spectrum shows one type of DMAP and one other carbon at 93.1 ppm. All attempts to find the other ring carbon failed. UV–vis spectroscopy showed λ_{max} at 314 nm (ϵ = 42 000). Elemental analysis was consistent with the assigned structure, assuming three waters of crystallization.

Crystal Structure of 3. Columnar orange-yellow crystals of 3 (BF_4^-) were obtained by slow crystallization from acetonitrile/water at room temperature and the structure was determined by X-ray crystallography. Figure 1 shows the ORTEP drawing of 3. Hydrogen atoms, where represented, are given as arbitrary small spheres. Each unit cell has a crystallographic center of inversion. The BF_4^- counterions are also related by the inversion center. Selected bond lengths and angles are given in Table I. One interesting feature is the dihedral angle of 54.9° between the pyridinium moieties and the benzene ring. This noncoplanarity understandably arises from steric interactions between the pyridinium ring and the ring fluorines. Both pyridinium rings are twisted in the same direction as seen in Figure 1. Note also the disorder shown by two of the methyl groups.

An interesting feature of the pyridinium moieties is that they have a substantial degree of quinoidal character. The bond length between the α- and β-carbons of the pyridinium ring is significantly shorter (1.34 Å) than the bond length between the β- and γ-carbons (1.41 Å). The γ-carbon-to-amino nitrogen distance, 1.33 Å, is between a carbon–nitrogen single and double bond, 1.47 and 1.27 Å, respectively, indicative of significant conjugation. Such conjugation has also been observed for other pyridinium compounds such as 1,2,3,3-tetrakis(4-(dimethylamino)-pyridinium-1-yl)cyclopropene[6] and methyl 3,3-bis(4-(dimethylamino)pyridinium-1-yl)propenoate dichloride.[7] Bond distances within the benzene ring are approximately equal, indicating no distortion of the ring due to the pyridinium groups. The BF_4^- counterions are held tightly between pyridinium groups of different molecules as shown in the unit cell in Figure 2.

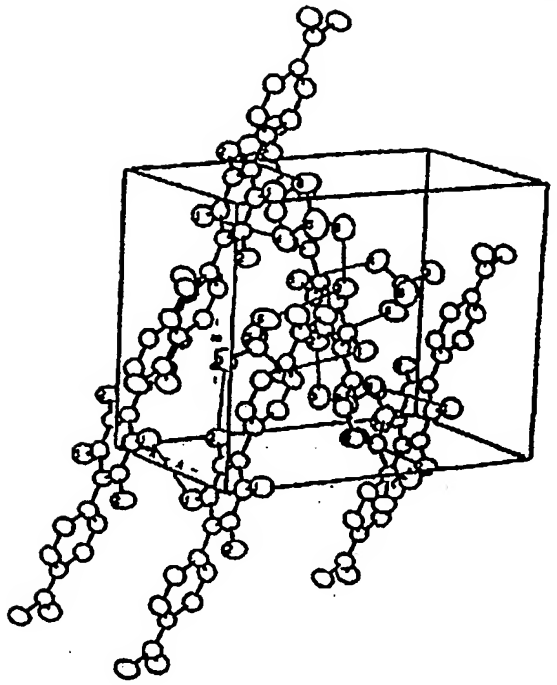

Figure 2. Unit cell of 3.

There are numerous close interactions between the gegenion fluorines and ring-bound fluorines, in addition to the relatively short distances of the ring-bound fluorines of two different molecules. The distance between two fluorines on different phenyl rings is 3.18 Å. There are no solvent molecules associated with the single crystal, unlike the chloride salt of methyl 3,3-bis(4-(dimethylamino)-pyridinium-1-yl)propenoate, in which the chloride ions are heavily solvated by water.[8]

Reaction of DMAP with hexafluorobenzene in more polar solvents gave highly substituted products. In refluxing acetonitrile a compound assigned as 1-fluoro-2,3,4,5,6-pentakis((4-dimethylamino)pyridinium-1-yl)benzene pentafluoride (4) was isolated in low yield from a black tarry product mixture. The reaction proceeded

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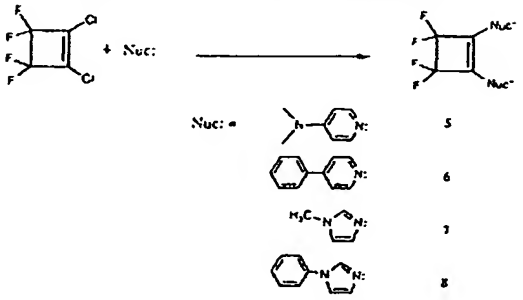
Scheme III. Nucleophilic Reactions of 1,2-Dichlorotetrafluorocyclobutene



slowly, taking several days before a significant amount of the black tarry precipitate formed that contained 15% of 4, based on the ^{1}H NMR spectrum. When 1,3-dimethyl-2-imidazolidinone (DMEU) or *N*-methyl-2-pyrrolidone (M-Pyrol) was used as the solvent, the reaction occurred facilely at room temperature but also produced more side products.

Assignments are based on the following data. Compound 4 has a λ_{max} at 297 nm, ϵ = 36 000, and a shoulder at 340 nm. The ^{1}H NMR spectrum shows three types of DMAP in a 1:2:2 ratio with no other protons, while its ^{19}F NMR spectrum indicates two types of fluorine. The broad-band decoupled ^{13}C NMR spectrum of 4 (BF_4^- salt) shows signals that are consistent with three different DMAP rings. The signal at 41.00 ppm is broad and unsymmetrical and is assumed to be two different dimethylamino groups overlapping. Elemental analysis of 4 was consistent with the assigned structure assuming one water molecule of crystallization.

Gram quantities of 3 were cleanly converted to 4 by dissolving 3 in a minimum amount of methanol to which was then added a solution of DMAP in acetonitrile or M-Pyrol. The resulting solution left to stand for several weeks gave a 36% yield of 4 after recrystallization. As the reaction proceeded, the disappearance of 3 and the appearance of 4 was followed by ^{1}H NMR spectroscopy, with no sign of the formation of other pyridinium compounds. Thus, the intermediate tri- and tetrasubstituted benzenes necessarily formed are clearly too reactive to accumulate in any appreciable amount. When the reaction of hexafluorobenzene with DMAP was carried out at a pressure of 15 KBar, other uncharacterized products containing pyridinium moieties were observed along with some 4. There was no sign of further addition of DMAP to 4, even when 4 and DMAP were heated to 60 °C at 15 KBar.

1,2-Dichlorotetrafluorocyclobutene. Nucleophiles have been found to react readily with 1,2-dichlorotetrafluorocyclobutene to give the mono- or disubstituted cyclobutene products.[9-11] The reactions of DMAP and 1-methylimidazole with 1,2-dichlorotetrafluorocyclobutene in acetonitrile at 0 °C produced two light yellow precipitates, 1,2-bis(4-(dimethylamino)pyridinium-1-yl)tetrafluorocyclobutene (5) and 1,2-bis(3-methylimidazolium-1-yl)tetrafluorocyclobutene (7), respectively (Scheme III). The ^{1}H NMR spectra of 5 and 7 show only one type of pyridinium and imidazolium groups. Both the ^{13}C decoupled NMR and ^{19}F NMR spectra of 5 and 7 are consistent with the assigned structures. Elemental analyses of the hexafluorophosphate salts of 5 and 7 show no waters of crystallization, whereas the dichloride salt of 5 has one such water. Similar reaction of 1,2-dichlorotetrafluorocyclobutene with 4-phenylpyridine in acetonitrile at 55 °C produced a yellow precipitate in 17% yield. Recrystallization from acetonitrile/diethyl ether produced 1,2-bis(4-phenylpyridinium-1-yl)tetrafluorocyclobutene (6) as yellow plates. The ^{1}H NMR and ^{19}F NMR spectra are consistent with the assigned structure. While both 5 and 7 readily precipitated out of solution, the synthesis and purification of 1,2-bis(3-phenylimidazolium-1-yl)tetrafluorocyclobutene (8) proved to be more difficult. Two crystallizations from diethyl ether/acetonitrile were needed to isolate 8 (BF_4^- salt) as an off-white waxy solid. Its ^{1}H NMR and ^{19}F NMR spectra and elemental analysis are consistent with the assigned structure, assuming two molecules of water.

Table II. Oxidation/Reduction Potentials vs SCE for 1–8 in Acetonitrile

compound (salt)	E^0_{oxid} (V)	E^0_{red} (V)
1 (BF_4^-)		−1.76
2 (BF_4^-)		−1.76
3 (BF_4^-)		−2.20, −1.85, −1.66, −1.10
4 (BF_4^-)		−2.65, −2.40, −2.23, −2.03, −1.24
5 (PF_6^-)	1.32, 1.58	−0.82, −1.02
5 (BF_4^-)		−0.82, −1.05
6 (PF_6^-)		−0.94, −0.79
7 (PF_6^-)		−0.75, −0.45
7 (BF_4^-)		−0.76, −0.52
8 (BF_4^-)		−1.15 V, −0.76

* Note that the electrochemical reaction conditions are described in the experimental General section.

Electrochemistry. The results of the electrochemical experiments performed on compounds 1–8 are shown in Table II. Neither 1 nor 2 could be oxidized electrochemically. Irreversible reduction of the pyridinium rings was observed at −1.76V vs SCE. Compound 3 was found to undergo four irreversible reductions at −2.20, −1.85, −1.66, and −1.10 V vs SCE. Since 3 undergoes four reductions and only has two pyridinium moieties, it seems reasonable to assume that the benzene ring is also undergoing reduction. These reductions were found to be irreversible even when scanning was done just up to the point of reduction. Compound 4 was found to undergo five irreversible reductions in acetonitrile with tetrabutylammonium tetrafluoroborate as the supporting electrolyte. Reductions occurred at −2.65, −2.40, −2.23, −2.03, and −1.24 V vs SCE. ($E^0 \simeq E_{1/2}$). Each of the substituted cyclobutenes 5–8 underwent irreversible reductions, which usually disappeared after the first five scans. The cyclobutenes tend to adhere to the glassy carbon electrode surface, so that eventually no reduction waves can be seen. It should be noted that the DMAP-substituted cyclobutene 5 (PF_6^- salt) also underwent two irreversible oxidations at 1.32 and 1.58 V vs SCE.

Conclusion

Highly halogenated alkenes and benzenes react with nucleophilic amines such as DMAP and N-substituted imidazoles to give multiply charged products. Many of these products, such as 1–8, undergo irreversible reductions in acetonitrile and show that the resulting radical ions are

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unstable to the reaction conditions. Accordingly, this result means that these structures have diminished promise as potential monomers for electron-conducting polymers and electrode coatings. Nevertheless, each of the cyclic systems presented is the first pyridinium- or imidazolium-substituted molecule of its type to be reported. They extend the generality of multiply charged compounds in which heterocyclic onium cation rings are joined by conjugating groups.

Experimental Section

General. General experimental procedures, spectroscopy, and materials are as described in our previous work.[6,7,12]

UV–visible spectra were determined with a Hewlett-Parkard 8452A diode array spectrophotometer, for which we thank Professor Mark Bednarski; results are expressed as (solvent), λ_{max}, in nm (extinction coefficient).

Gas chromatography (GC) was done with a Hewlett-Packard series 5880 gas chromatograph, with a 0.125 in., 6 ft, SE 30 on Chromosorb column. Thin-layer chromatography (TLC) was performed in 50% hexane/50% ethyl acetate or 100% ethyl acetate. The buffer solutions were calibrated with a Fisher Accumet Model 805 MP pH meter.

Columnar orange-yellow single crystals of 3, suitable for X-ray analysis, were grown by slow evaporation from acetonitrile/water. Preliminary cell constants and space-group analyses were obtained from precession photographs on an Enraf-Nonius precession camera. Data collection was performed on a CAD4 diffractometer at room temperature. Data were collected by monitoring three intensity standards (4 3 3, 2 7 2, 1 4 $\bar{8}$) every 1 h and by checking three orientation standards (4 3 3, 2 7 2, 1 4 $\bar{8}$) every 200 reflections. Crystal orientation was redetermined if any of the reflections were offset by more than 0.10° from their predicted positions. Reorientation was not needed during data collection.[13] A total of 1606 raw intensity data were collected which were converted to structure factor amplitudes and their ESD's by correction for scan speed, background, and Lorentz and polarization effects. Inspection of the intenstiy standards revealed a reduction of 4% of the original intensity. The data were corrected for this decay. Inspection of the azimuthal scan data showed a random variation $I_{min}/I_{max} = \pm 1\%$ for the average curve. No correction for absorption was applied. Inspection of the systematic absences indicated uniquely space group $P2_1/n$. Removal of systematically absent data left 1506 unique data in the final set. Structure of 3 was solved by direct methods (MULTAN) and refined via standard least-squares and Fourier techniques. Peaks corresponding to the positions of all of the hydrogen atoms were calculated following the refinement of all non-hydrogen atoms with anisotropic thermal parameters in a difference Fourier map. Hydrogen atoms were included in the structure at their calculated positions with d(C–H) = 0.95 Å and with an isotropic thermal parameter 1.2 times the equivalent isotropic thermal parameter of the carbon to which they are attached. The final cycle of least-squares refinement yielded $R = 2.98\%$, $R_w = 4.32\%$, and GOF = 2.19. The R value for all 1506 data was 4.00%.

Cyclic voltammetry was performed on a EG&G Princeton Applied Research (PAR) Model 173 potentiostat/galvanostat, with a Model 176 current follower, driven by a Model 175 universal programer. Cyclic voltammograms were recorded on a Houston Instruments Model 200 XY recorder. For compounds 1–4, best results were obtained using glassy carbon for both the working and counter electrodes. For compounds 5–8, the working electrode was glassy carbon, while the counter electrode was platinum wire. A silver wire placed in a capillary tube to minimize contact with any decomposition products was used for the pseudo-reference electrode for all samples. Aqueous samples were run at a concentration of about 10 mM in 0.1 M KCl as a supporting electrolyte. Samples 1–4 run in acetonitrile used either 0.1 M tetrabutylammonium tetrafluoroborate or 0.1 M tetrabutylammonium hexafluorophosphate. Samples 5–8 run in acetonitrile used either 0.25 M tetrabutylammonium tetrafluoroborate or 0.25 M tetrabutylammonium hexafluorophosphate as the electrolyte. All nonaqueous potentials were referenced to ferrocene as an internal standard. Multiple scans were recorded for each compound with little or no variation between scans.

Prior to submission for elemental analyses, all samples were weighed in tared vials and dried for at least 24 h under a minimum vacuum of 0.01 mmHg. All samples in the vials were then stored in argon, which was first passed through a drying tube of fresh Drierite. Elemental analyses were performed by the Microanalytical Laboratory, operated by the College of Chemistry, University of California, Berkeley, CA.

1,2,3,4,5-Pentakis(4-(dimethylamino)pyridinium-1-yl)cyclopentadienyl Anion Pentachloride (1) and 1-Chloro-2,3,4,5-tetrakis(4-(dimethylamino)pyridinium-1-yl)cyclopentadienyl Anion Tetrachloride (2). To a solution of 0.77 g (2.83 mmol) of hexachlorocyclopentadiene in 5 mL of acetonitrile was added a solution of 4.14 g (33.92 mmol) of 4-(dimethylamino)-pyridine (DMAP) in 70 mL of acetonitrile. The reaction mixture was kept for 100 h at 82 °C. Upon addition of 5 drops of water a brownish precipitate formed. The precipitate was collected via suction filtration and reprecipitated from methanol with acetone. The solid was collected again via suction filtration, dissolved in a minimal amount of methanol, and reprecipitated slowly from a 50/50 mixture of 2-propanol and *tert*-butyl alcohol to give 237 mg (0.331 mmol, 12%) of 2; mp 233 °C dec: ^{1}H NMR (250 MHz, D_2O, CH_3CN internal standard) δ 3.03 (12 H, s), 3.09 (12 H, s), 6.64 (4 H, d, J = 7.8 Hz), 6.76 (4 H, d, J = 7.8 Hz), 7.80 (4 H, d, J = 7.8 Hz), 7.87 (4 H, d, J = 7.8 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 157.7, 157.6, 145.5, 114.2, 112.2, 109.1, 108.9, 97.0, 40.6; IR (KBr), 3050 (b, m), 2625 (w), 1630 (sh, s), 1570 (b, s), 1440 (w), 1400 (sh, s), 1340 (w), 1210 (b, s), 1150 (sh, s), 1080 (sh, w), 1060 (w), 1020 (w), 970 (sh, w), 935 (sh, w), 820 (sh, s), 755 (sh, w); UV–vis (H_2O) λ 305.6 (ϵ 58 600); (concd H_2SO_4) λ 298.4, 335 shoulder, 385 shoulder.

Anal. Calcd for $C_{30}H_{40}N_8Cl_4 \cdot 4H_2O$: C, 51.97; H, 6.34; N, 14.69; Cl, 18.60. Found: C, 52.25; H, 6.53; N, 14.62; Cl, 19.31.

The mother liquor from the first reprecipitation of 2 was evaporated to dryness. The resulting solid was dissolved in a minimal amount of methanol and slowly precipitated from a 50/50 mixture of 2-propanol and *tert*-butyl alcohol to give 639 mg (0.715 mmol, 25%) of 1; mp 281 °C dec: ^{1}H NMR (250 MHz, D_2O, CH_3CN internal standard) δ 3.06 (30 H, s), 6.67 (10 H, d, J = 7.8 Hz), 7.86 (10 H, d, J = 7.8 Hz); (D_2SO_4, no standard). The NMR spectrum of the yellow solution shows four types of pyridinium, one being very weak. Upon dilution with 4 equiv of D_2O, the solution became colorless and showed only one type of pyridinium: ^{1}H NMR (250 MHz, D_2O, CH_3CN internal standard) δ 3.00, (30 H, s), 6.61 (10 H, d, J = 6.3 Hz), 7.67 (10 H, d, J = 6.3 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 157.7, 145.4, 112.6, 109.0, 40.7; IR (KBr) 3010 (b, m), 2630 (w), 1630 (b, m), 1580–1510 (b, s), 1400 (sh, s), 1340 (w), 1320 (w), 1220 (sh, s), 1150 (sh, s), 935 (sh, m), 830 (sh, s); UV–vis (H_2O) λ 308 (ϵ = 83 600); (concd H_2SO_4) λ 300, 340 shoulder, 400 shoulder.

Anal. Calcd for $C_{40}H_{50}N_{10}Cl_4 \cdot 4.5H_2O$: C, 53.75; H, 6.65; N, 15.67; Cl, 15.87. Found: C, 53.72; H, 6.52; N, 15.60; Cl, 16.49.

Acidity Determination of 1. A standard base solution was prepared by diluting 5.66 g (0.101 mol) of KOH to 100 mL and diluting 50 mL of this solution to 500 mL. Concentration of KOH solution was determined by a three-point titration with potassium acid phthalate. NMR solutions were prepared by diluting concd D_2SO_4 with D_2O, and molarity was determined by three-point titration using the above standard base solution. Since no standard is known for D_2SO_4, the strongest upfield absorption was set to 3.0 ppm. Peaks were severely broadened, and spectra were obtained in 17.5, 14.4, 13.6, 12.8, and 11.7 M D_2SO_4.

1,2,4,5-Tetrafluoro-3,6-bis((4-dimethylamino)pyridinium-1-yl)benzene Difluoride (3a). To a solution of 0.51 g (2.77 mmol) of hexafluorobenzene in 3 mL of THF was added 2.03 g

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(13) Diffraction data for the crystal structure was collected by Dr. Fred Hollander, director of the UCB X-ray facility. The authors have deposited atomic coordinates for this structure with the Cambridge Crystallographic Data Centre. The coordinates can be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, UK.

(16.61 mmol) of DMAP in 40 mL of THF. The reaction mixture was stirred at reflux under a N_2 atm for 24 h and cooled to room temperature, and the resulting yellow precipitate was collected via suction filtration to give 0.32 g of crude product. Its ^1H NMR spectrum showed the product to be about 56% 3 (NMR yield, 13%). The crude material was reprecipitated from methanol, acetone, and THF to give 0.073 g of pure 3 (5% yield). The original reaction mixture was refluxed for 48 h to give 0.47 g of a yellowish brown solid which proved to be 22% 3 by ^1H NMR spectroscopy; NMR yield 7.7%. When the reaction was allowed to proceed for more than 24 h, 3 underwent further reaction to give a variety of higher substituted products. ^1H NMR (250 MHz, D_2O, CH_3CN internal standard): δ 3.23 (12 H, s), 7.00 (4 H, d, J = 8.0 Hz), 8.03 (4 H, d, J = 7.6 Hz). ^{19}F NMR (250 MHz, CD_3OD, $CFCl_3$ internal standard): δ −146.5, −164.8. UV–vis (H_2O): λ 314 (ϵ = 42 000).

Anal. Calcd for $C_{20}H_{20}N_4F_6{\cdot}3H_2O$: C, 49.59; H, 5.41; N, 11.57. Found: C, 49.89; H, 4.92; N, 11.28.

1,2,4,5-Tetrafluoro-3,6-bis((4-dimethylamino)pyridinium-1-yl)benzene Bis(tetrafluoroborate) (3b). The X-ray single crystal structure of 3b was obtained by first dissolving 3a in a minimal amount of water and acetonitrile. Excess sodium tetrafluoroborate was added to the solution. To keep the solution free of dust particles, the vial was then covered with a Kimwipe tissue, fastened with a rubber band. Over a period of several days, acetonitrile slowly evaporated to afford crystals of 3b in the remaining aqueous solution.

1-Fluoro-2,3,4,5,6-pentakis((4-dimethylamino)pyridinium-1-yl)benzene Pentafluoride (4a). Under High Pressure. A solution of 0.21 g (1.12 mmol) of hexafluorobenzene and 0.75 g (6.15 mmol) of DMAP in 8 mL of 1:3 (v:v) THF–CH_3CN was placed in a 10-mL syringe. The end was sealed with a hot spatula and the plunger arm was cut off. The syringe was then pressed to 15 Kbar for 4 h in a high pressure piston press.[14] The syringe was then allowed to stand at room pressure for 4 d before collecting 0.24 g of brown solid via suction filtration. Precipitation from a mixture of methanol, acetonitrile, and THF was followed by multiple recrystallizations from methanol and 2-propanol to give 0.072 g (0.090 mmol, 8%) of 4: ^1H NMR (250 MHz, CD_3OD, CH_3CN internal standard) δ 3.04 (6 H, s), 3.06 (12 H, s), 3.11 (12 H, s), 6.67 (2 H, br d), 6.72 (4 H, d, J = 7.5 Hz), 6.82 (4 H, d, J = 7.6 Hz), 7.88 (10 H, complex mult); ^{19}F NMR (250 MHz, CD_3OD, $CFCl_3$ internal standard) δ −130, −149; UV–vis (H_2O) λ 297 (ϵ 36,000), 340 nm shoulder.

Thermally. A 27% THF in acetonitrile solution containing 0.70 g (3.78 mmol) of hexafluorobenzene and 2.82 g (23.1 mmol) of DMAP was heated at reflux for 2 h and allowed to stand at room temperature for 4 days. Filtering the solution gave 0.25 g of a black solid which appeared to be mostly 4 by ^1H NMR spectroscopy. Addition of 4 drops of water to the mother liquor produced 0.28 g of an off-white precipitate that appeared to be a mixture of 4 and other pyridinium-substituted benzenes. Multiple recrystallizations from methanol and 2-propanol gave 14 mg (0.5%) of 4.

1-Fluoro-2,3,4,5,6-pentakis((4-dimethylamino)pyridinium-1-yl)benzene Pentakis(tetrafluoroborate) (4b). From 3. To a solution of 1.00 g (2.32 mmol) of 3 in 8 mL of methanol was added a solution of 1.13 g (9.29 mmol) of DMAP in 35 mL of acetonitrile. The reaction mixture was sealed and allowed to stand at rt for 3.5 months. The resulting solid was collected via suction filtration and washed with 3 × 2 mL of acetonitrile and 3 × 5 mL of acetone to give 1.64 g (2.01 mmol) of 4, yield 80%. Fluoride ions were exchanged for tetrafluoroborate ions by dissolving the sample in a minimum amount of water and adding a solution of 1.55 g (14.1 mmol) of $NaBF_4$ in 30 mL of water. The resulting precipitate was collected via suction filtration and reprecipitated from acetonitrile/water: yield, 1.05 g (0.835 mmol, 36%); mp 285–355 °C dec; ^1H NMR (250 MHz, CD_3OD, CH_3CN internal standard) δ 3.04 (6 H, s), 3.06 (12 H, s), 3.11 (12 H, s), 6.67 (2 H, br d), 6.72 (4 H, d, J = 7.5 Hz), 6.82 (4 H, d, J = 7.6 Hz), 7.88 (10 H, complex mult); ^{19}F NMR (250 MHz, CD_3OD, $CFCl_3$ internal standard) δ −130, −149; UV–vis (H_2O) λ 297 (ϵ = 36 000), 340 shoulder; ^{13}C NMR (75 MHz, CD_3CN) δ 164.57, 157.40, 157.30, 157.22, 143.92, 143.77, 141.77, 137.67, 132.34, 114.06, 109.60, 109.05, 41.01, 40.96, 40.82; ^{13}C DEPT (100 MHz, CD_3CN) 135° δ 143.92, 143.77, 141.77, 109.60, 109.05, 41.01, 40.96, 40.82.

Anal. Calcd for $C_{41}H_{50}N_{10}B_3F_{21}{\cdot}H_2O$: C, 42.68; H, 4.54; N, 12.14; B, 4.68. Found: C, 42.52; H, 4.83; N, 12.58; B, 4.95.

1,2-Bis(4-dimethylamino)pyridinium-1-yl)tetrafluorocyclobutene Dichloride (5a). To a stirring solution of 1.48 (7.59 mmol) of 1,2-dichlorotetrafluorocyclobutene in dichloromethane (50 mL) was added 1.78 g (14.6 mmol) of DMAP over a 3-h period. The reaction mixture immediately turned yellow, and a yellow solid precipitated. As the DMAP was added, more precipitate formed at the bottom of the flask. Any unreacted 1,2-dichlorotetrafluorocyclobutene and dichloromethane solvent were removed by rotary evaporation. The remaining yellow solid was dried overnight under vacuum; yield, 2.1 g (63%); mp 103–105 °C; ^1H NMR (300 MHz, D_2O, CH_3CN internal standard) δ 7.95 (d, 4, J = 8.0 Hz), 7.01 (d, 4, J = 8.0 Hz), 3.28 (s, 12); ^{19}F NMR (250 MHz, D_2O, $CFCl_3$ internal standard) δ −114.07; ^{13}C NMR (75.8 MHz, D_2O, CH_3OH internal standard) δ 165.1, 146.0, 145.8, 145.6, 117.6, 117.5, 117.3, 49.0.

Anal. Calcd for $C_{18}H_{20}N_4F_4Cl_2{\cdot}H_2O$: C, 47.28; H, 4.85; N, 12.25. Found: C, 47.60; H, 4.69; N, 12.13.

1,2-Bis(4-(dimethylamino)pyridinium-1-yl)tetrafluorocyclobutene Bis(hexafluorophosphate) (5b). To a stirring solution of 1.49 g (7.64 mmol) of 1,2-dichlorotetrafluorocyclobutene in acetonitrile (25 mL) was added 1.82 g (14.9 mmol) of DMAP over a 3-h period. The reaction mixture immediately turned yellow, and an orange solid precipitated. As the DMAP was added, more precipitate formed at the bottom of the flask. The yellow mixture was stirred at rt under N_2 for 9 h. Any unreacted 1,2-dichlorotetrafluorocyclobutene and acetonitrile solvent were removed by rotary evaporation. The remaining orange solid was dissolved in water (25 mL), to which was added a solution of 2.69 g (16.0 mmol) of $NaPF_6$ in water (10 mL). The resulting yellow precipitate was vacuum filtered and dried overnight under vacuum: yield, 4.27 g (85%); ^1H NMR (400 MHz, CD_3CN) δ 7.87 (dd, 4, J = 8.0, 2.3 Hz), 7.03 (dd, 4, J = 8.0, 2.1 Hz), 3.34 (s, 12); ^{19}F NMR (400 MHz, CD_3CN, $CDCl_3$ internal standard) δ −1.87, −42.94; ^{13}C NMR (100 MHz, CD_3CN) δ 158.0, 138.8, 118.4, 110.5, 42.0.

Anal. Calcd for $C_{18}H_{20}N_4P_2F_{16}$: C, 32.84; H, 3.06; N, 8.51. Found: C, 32.66; H, 2.95; N, 8.56.

1,2-Bis(4-(dimethylamino)pyridinium-1-yl)tetrafluorocyclobutene Bis(tetrafluoroborate) (5c). Hexafluorophosphate ions were exchanged for tetrafluoroborate ions by dissolving 5b in a minimum amount of acetonitrile and running it down a column of Amberlyst A-21 resin with 50% aqueous methanol as eluant. (The Amberlyst A-21 column had been previously flushed three times with an aqueous solution of saturated sodium tetrafluoroborate.) After the yellow solution was collected from the column, methanol was removed by rotary evaporation, and water was removed by lyophilization. The resulting yellow solid was dried overnight under vacuum: ^1H NMR (300 MHz, CD_3CN) δ 7.89 (d, 4, J = 0.75 Hz), 7.04 (t, 4, J = 1.8 Hz), 3.34 (s, 12); ^{19}F NMR (400 MHz, CD_3CN, $CDCl_3$ internal standard) δ −114.1, −151.7.

1,2-Bis(4-phenylpyridinium-1-yl)tetrafluorocyclobutene Dichloride (6). To a stirring solution of 0.307 g (1.57 mmol) of 1,2-dichlorotetrafluorocyclobutene in acetonitrile (25 mL) was added 0.49 g (3.16 mmol) of 4-phenylpyridine. The reaction was stirred under N_2 at 55 °C for 12 h. Upon cooling, yellow needles precipitated. The crystals were filtered and dried in vacuo to yield 0.127 g (16%) of product. Crystallization from acetonitrile/diethyl ether afforded 0.105 g of yellow plates: mp 324–325 °C dec; ^1H NMR (300 MHz, acetone-d_6) δ 8.82 (2 H, dd, J = 1.53, 5.26 Hz), 8.26 (2 H, dd, J = 1.57, 5.25 Hz), 7.97 (2 H, ddd, J = 2.12, 3.51, 4.75 Hz), 7.62 (3 H, m); ^{19}F NMR (400 MHz, CD_3CN, $CFCl_3$ internal standard) δ −115.6.

1,2-Bis(3-methylimidazolium-1-yl)tetrafluorocyclobutene Dichloride (7a). To a stirring solution of 1.22 g (6.24 mmol) of 1,2-dichlorotetrafluorocyclobutene in acetonitrile (25 mL) was added 1.17 g (14.2 mmol) of 1-methylimidazole. The reaction solution was stirred at rt under a N_2 atm and turned from pale yellow to orange. After 2 min, a yellow precipitate formed. After vacuum filtration, the yellow solid was dissolved

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in a minimal amount of methanol, precipitated with acetone to form a cloudy mixture, and then redissolved with methanol to form a clear solution. After chilling the solution overnight, white crystals were isolated by vacuum filtration: yield, 1.6 g (70%); mp 134–136 °C; ^{1}H NMR (300 MHz, D_2O, CH_3CN internal standard) δ 9.64 (s, 2), 7.84 (d, 2, J = 2.2 Hz), 7.75 (d, 2, J = 2.2 Hz), 3.96 (s, 6); ^{19}F NMR (250 MHz, D_2O, $CFCl_3$ internal standard) δ −115.01; ^{13}C NMR (75.8 MHz, D_2O, CH_3CN internal standard) δ 126.9, 126.8, 126.7, 126.54, 126.49, 121.73, 121.71, 115.0, 37.4.

Anal. Calcd for $C_{12}H_{12}N_4F_4Cl_2$: C, 40.13; H, 3.37; N, 15.60. Found: C, 39.98; H, 3.39; N, 15.45.

1,2-Bis(3-methylimidazolium-1-yl)tetrafluorocyclobutene Bis(hexafluorophosphate) (7b). To a solution of 1.22 g (6.24 mmol) of 1,2-dichlorotetrafluorocyclobutene in acetonitrile (25 mL) was added 1.17 g (14.2 mmol) of 1-methylimidazole. After 3 min, a yellow precipitate formed. The reaction solution was stirred for 9 h at rt under N_2. Rotary evaporation was used to removed excess starting reagents and acetonitrile. The yellow solid was dissolved in water (15 mL), to which was added a solution of 2.19 g (13.1 mmol) of $NaPF_6$ in water (15 mL). After the PF_6^- salt precipitated, it was vacuum filtered and dried overnight under vacuum: yield, 3.17 g (88%); ^{1}H NMR (400 MHz, CD_3CN) δ 9.04 (s, 2), 7.70 (t, 2, J = 2.2 Hz), 7.64 (t, 2, J = 2.2 Hz), 2.85 (s, 6); ^{19}F NMR (400 MHz, CD_3CN, $CDCl_3$ internal standard) δ −115.05, −73.24, −71.36; ^{13}C NMR (100 MHz, CD_3CN) δ 138.5, 127.5, 122.3, 38.2.

Anal. Calcd for $C_{12}H_{12}N_4P_2F_{16}$: C, 24.93; H, 2.09; N, 9.69. Found: C, 24.55; H, 2.11; N, 9.89.

1,2-Bis(3-methylimidazolium-1-yl)tetrafluorocyclobutene Bis(tetrafluoroborate) (7c). Hexafluorophosphate counterions were exchanged for tetrafluoroborate ions as previously described for 5c. The resulting yellow solid was dried overnight under vacuum: ^{1}H NMR (300 MHz, CD_3CN) δ 8.81 (s, 2), 7.57 (dd, 4, J = 1.8, 7.1 Hz), 3.89 (s, 6); ^{19}F NMR (400 MHz, CD_3CN, $CFCl_3$ internal standard) δ −71.5, −73.3, −152.06.

1,2-Bis(3-phenylimidazolium-1-yl)tetrafluorocyclobutene Bis(tetrafluoroborate) (8). To a solution of 1.11 g (5.67 mmol) of 1,2-dichlorotetrafluorocyclobutene in 80% diethyl ether/20% CH_2Cl_2 (25 mL) was added 1.68 g (11.7 mmol) of 1-phenylimidazole. After 1 h of stirring at 0 °C, the reaction solution was allowed to stand at 0 °C overnight. Clusters of an off-white precipitate were removed by vacuum filtration. Because the dichloride salt was extremely hygroscopic, the solid was immediately converted to the tetrafluoroborate salt. The sample (1.10 g, 2.28 mmol) was dissolved in a minimal amount of water, to which was added a solution of 0.51 g (4.62 mmol) of $NaBF_4$ in 15 mL of water. After the BF_4^- salt precipitated, it was vacuum filtered and dissolved in a minimal amount of acetonitrile. Drops of diethyl ether were added until the solution became cloudy, and then drops of acetonitrile were added to produce a clear solution again. The solution was chilled at 0 °C for 9 h to produce a light yellow precipitate. This solid was vacuum filtered and recrystallized from acetonitrile/diethyl ether as previously described. The off-white waxy solid was vacuum filtered and dried overnight under vacuum: yield, 0.56 g (17%); ^{1}H NMR (400 MHz, CD_3CN) δ 8.97 (s, 2), 7.77 (t, 2, J = 2.1 Hz), 7.62 (t, 2 J = 2.2 Hz), 7.55 (d, 4, J = 7.1 Hz), 7.43 (m, 6); ^{19}F NMR (400 MHz, CD_3CN, $CFCl_3$ internal standard) δ −142.09, −77.33.

Anal. Calcd for $C_{22}H_{16}N_4B_2F_{12}\cdot 2H_2O$: C, 42.48; H, 3.24; N, 9.01. Found: C, 42.79; H, 3.47; N, 8.77.

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Supplementary Material Available: All available ^{1}H NMR, ^{13}C NMR, and ^{19}F NMR spectra for the 1,2,3,4,5-pentakis(4-(dimethylamino)pyridinium-1-yl)cyclopentadienyl anion (1), 1-chloro-2,3,4,5-tetrakis(4-(dimethylamino)pyridinium-1-yl)cyclopentadienyl anion (2), 1,2,4,5-tetrafluoro-3,6-bis(4-(dimethylamino)pyridinium-1-yl)benzene (3), 1-fluoro-2,3,4,5,6-pentakis-(4-(dimethylamino)pyridinium-1-yl)benzene (4), 1,2-bis(4-(dimethylamino)pyridinium-1-yl)tetrafluorocyclobutene (5), 1,2-bis-(4-phenylpyridinium-1-yl)tetrafluorocyclobutene (6), 1,2-bis(3-methylimidazolium-1-yl)tetrafluorocyclobutene (7), and 1,2-bis(3-phenylimidazolium-1-yl)tetrafluorocyclobutene (8) (35 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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DNA sequencing with dye-labeled terminators and T7 DNA polymerase: effect of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments

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ABSTRACT

Th incorporation of fluorescently labeled dideoxynucleotides by T7 DNA polymerase is optimized by the use of Mn^{2+}, fluorescein analogs and four 2'-deoxyribonucleoside 5'-*O*-(1-thiotriphosphates) (dNTPαS's). The one-tube extension protocol was tested on single-stranded templates, as well as PCR fragments which were made single-stranded by digestion with T7 gene 6 exonuclease. Dye primer sequencing using four dNTPαS's was shown to give uniform termination patterns which were comparable to four dNTPs. Efficiency of the polymerase also appeared to improve with the dNTPαS's. A mathematical model was developed to predict the pattern of termination based on enzyme activity and ratios of ddNTP/dNTPs. This method can be used to optimize sequencing reactions and to estimate enzyme discrimination constants of chain terminators.

INTRODUCTION

Sanger dideoxy DNA sequencing[1] has proved to be the most durable and efficient method of DNA sequencing and is the method of choice of most investigators in large scale sequencing programs.[2] 'Automated' or 'fluorescent' DNA sequencing refers to a variation of traditional Sanger sequencing in which fluorescent labels are covalently attached to the reaction products and data is collected during the polyacrylamide gel electrophoresis. Fluorescent sequencing can be divided into two categories: 'dye primer' sequencing, in which case the fluorescent dyes are attached to the 5' end of the primer, and 'dye terminator' sequencing, in which case the fluorescent dyes are attached to the dideoxynucleoside triphosphates. Several companies (Applied Biosystems (ABI)[3,4], du Pont de Nemours & Co (Du Pont)[5], Pharmacia LKB Biotechnology Inc.[6], and Hitachi[7]) have produced automated DNA sequencers based on fluorescent dyes using dye primer and dye terminator sequencing.

In dye primer sequencing, four separate extension/termination reactions are carried out; each reaction contains four deoxynucleoside triphosphates (dNTPs) and one dideoxynucleoside triphosphate (ddNTP). In the ABI primer system, four separate dye primers are used, corresponding to each ddNTP. After the extension reaction, the four reactions are pooled and the nested set of oligonucleotide fragments are separated by electrophoresis. The dyes, and therefore the nucleotides, are distinguished from each other by their fluorescence emission spectra. In the Pharmacia system, a single dye primer is used in each of the four separate extension reactions. The reactions are not pooled but are run in four separate lanes in direct analogy to traditional, radioactive Sanger sequencing.

In dye terminator sequencing, the fluorescent label is incorporated into the DNA fragments in a single extension/termination reaction using an unlabeled primer. The products are separated, as with dye primer sequencing, by electrophoresis. Since the reaction is performed in one tube, both the Du Pont and the ABI terminator systems require four, spectrally resolvable fluorescent dyes.

There are advantages and disadvantages to both radioactive and fluorescent sequencing, and within fluorescent sequencing, between dye primer and dye terminator sequencing. Radioactive sequencing has the advantage of good signal-to-noise and a wide range in the amount of template needed for sequencing. The label can be incorporated many times in each oligonucleotide fragment during the extension by using radioactively-labeled dNTPs and the signal can be improved by longer film exposure times. Radioactive sequencing has the disadvantage of tedium, especially in the film analysis task, and the limitations in sequence ordering imposed by the requirement of combining data from four separate lanes on the polyacrylamide gel.

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Fluorescent sequencing has the advantages of automated data collection and analysis and of high throughput. It has the disadvantage of high capital cost and, in all of the currently available chemistries, of incorporating a single fluorescent label in each oligonucleotide fragment. The availability of *Thermus aquaticus* (taq) polymerase and cycle sequencing[8], however, has improved the range of the dye primer and dye terminator chemistries by linear amplification of the template and therefore, linear amplification of the signal.

Dye primers and dye terminator systems have their own set of advantages and disadvantages. One advantage of the dye primer system is that any DNA polymerase can be used as long as it accepts dideoxynucleoside triphosphates as substrates. The main disadvantage is the requirement for four separate extension reactions. In the DNA sequencing chemistry labor, dye-primers confer no time-saving advantage for fluorescent vs. manual sequencing.

Dye terminators offer the advantage of convenience. Synthesis of labeled primer is unnecessary, thereby allowing the use of any sequencing primer. Additionally, because only one extension reaction is needed for each sequence, the labor required to perform the extension reactions is reduced. Another advantage of this chemistry is that noise from 'false terminations', in which the oligonucleotide is terminated by a deoxynucleotide rather than a dideoxynucleotide, is eliminated, since such fragments are unlabeled and, therefore, invisible. A disadvantage of the chemistry is that each set of dye-labeled terminators must be tailored to a specific DNA polymerase. For any given enzyme, the pattern of termination from the sequence-dependent substrate specificities of the dideoxynucleotides tends to be less uniform for dye-labeled ddNTPs than for unlabeled ddNTPs. For a given amount of signal, uneven patterns cause increased noise, particularly in the underrepresented populations of oligonucleotide fragments (small peaks).

We previously have developed a set of dye-labeled terminators for taq DNA polymerase which utilized the same 3-amino-1-propyn-1-yl linker arm to attach the dye to the nucleotide as the original Du Pont system (Fig. 1). The Du Pont system utilizes a set of four dyes, each with a net −1 charge, and optimally uses T7 DNA polymerase with Mg^{2+}. The ABI taq terminators utilizes a set of four, zwitterionic rhodamine dyes[9]. Many variations on the dyes were made and tested with taq DNA polymerase, including varieties of fluorescein dyes, boron dipyridyl 'bodipy' dyes, and cyanine dyes. Rhodamine dyes were found to give the most acceptable results. Fluorescein dyes were poor substrates for taq polymerase. While the hydrophobicity of the rhodamine dyes appears to serve the dye-terminator well in the binding pocket of taq polymerase, the same characteristic causes problems in the gel electrophoresis. The rhodamine terminators cause 'compressions' in the data. That is, rhodamine terminators appear to stabilize hairpin structures in GC-rich regions, causing those fragments to migrate anomalously. The compression problem is entirely relieved by substituting 2'-deoxyinosine 5'-triphosphate (dITP) for deoxyguanosine triphosphate (dGTP). The use of dITP, however, results in a less even pattern of termination than results when dGTP is used.

This paper will focus on the development of a set of dye-labeled terminators for modified T7 DNA polymerase, a DNA polymerase developed by Tabor and Richardson[10] which is widely used in radioactive sequencing. The main advantage of T7 DNA polymerase over other DNA polymerases is its processivity and the striking evenness of termination patterns when manganese ions are used as a cofactor[11]. The addition of pyrophosphatase and Mg^{2+} makes the polymerase reaction essentially irreversible and solves the problem of disappearing peaks[12]. The main disadvantage is its lack of thermal stability. Therefore, for automated sequencing, the amplification of signal which is produced in taq cycle sequencing methods is unavailable. The purpose of developing a set of dye-terminators for T7 DNA polymerase was to capitalize on the characteristic processivity of the enzyme and to allow the use of any user-defined sequencing primer with the automated fluorescent sequencing instrument. The use of these dye terminators should provide another useful tool in the methods available for fluorescent sequencing.

MATERIALS AND METHODS

Instrumentation. Absorption spectra were obtained on an Hewlett-Packard Model 8451A UV-visible spectrophotometer. Fluorescence spectra were obtained on a Perkin-Elmer Model LS-5 fluorescence spectrophotometer. HPLC was performed using a Perkin-Elmer Series 4 Liquid Chromatograph. Columns (AX300, RP300) were obtained from Applied Biosystems, Inc. (ABI). PCR was performed on a Perkin-Elmer Cetus DNA thermal cycler or model 9600 DNA thermal cycler. DNA sequencing was performed on an ABI Model 373 DNA sequencer. Agarose gels were analyzed on an ABI Model 362 GENESCANNER.

DNA, oligonucleotides, nucleotides and enzymes. M13mp18 was obtained from Promega (Madison, WI). Oligonucleotides were prepared on an ABI Model 380B DNA synthesizer by Bill Guisti (ABI, Foster City, CA). The primers used were −21 primer (5'-TGTAAAACGACGGCCAGT), −40 primer (5'-GTTTTCCCAGTCACGAC) and reverse primer (5'-CAGGAAACAGCTATGACC). Nucleotides were obtained from Pharmacia LKB Biotechnology Inc., with the exception of (S_p)-dITPαS, which was a generous gift from Professor Fritz Eckstein (Max Planck Institut fur Experimentelle Medizin, Gottingen, W. Germany). The diasteriomeric mixture of dITPαS was synthesized by the method of Ludwig and Eckstein[13]. Sequenase© Version 2.0 T7 DNA polymerase, pyrophosphatase and T7 gene 6 exonuclease were obtained from United States Biochemical Corp. (Cleveland, OH). Sequenase© Dye-Primer sequencing kits were also obtained from USB. Pyrophosphatase was also obtained from Sigma Chemical Co. (St. Louis, MO). Amplitaq DNA polymerase was obtained from Cetus Corp. (Emeryville, CA).

Organic reagents. Tricarballylic acid, methanesulfonic acid, *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, 4-chloro-1,3-dihydroxybenzene and 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Fluorescent dyes. Bodipy dyes, bifluor, 5-carboxy-2',7'-dichlorofluorescein, 5-carboxy-2',4',5',7'-tetrachlorofluorescein (ZOE), 5-(and 6-)carboxy rhodamine 110, 6-carboxytetramethyl rhodamine and 6-carboxy rhodamine X were provided by Molecular Probes (Eugene, OR). The synthesis of the dyes 5-(and 6-)carboxy-4,7-dichloro-2',7'-dimethoxyfluorescein (BUB), 5-(and 6-)carboxy-4,7,4',5'-tetrachloro-2',7'-dimethoxyfluorescein (LOU), 5-(and 6-)carboxy-4,7-dichlorofluorescein

(4,7-DCF), and 5-(and 6-)carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) were originally described by Khanna and Ullman[14] and were synthesized at ABI with some modifications. The dyes 5-(and 6-)carboxy-4,7-dichloro-1',2',7',8'-dibenzofluorescein (NAN) and 5-(and 6-)carboxy-4,7,4',5'-tetrachloro-1',2',7',8'-dibenzofluorescein (DEB) and their 5-(and 6-)succinimidyl esters were synthesized at ABI by methods described elsewhere. For the dyes which have been characterized by NMR (FAM, JOE, all of the rhodamines), the 6-carboxy isomer was the faster-eluting isomer on reverse-phase HPLC columns. The uncharacterized dyes are designated as 'dye1' for the faster-eluted isomer or 'dye2' for the slower-eluting isomer. We believe the 6-carboxy isomer corresponds to 'dye1' and the 5-carboxy isomer to 'dye2'.

Synthesis of 9-(2',3'-dicarboxypropyl)-6-hydroxy-3H-xanthen-3-one (EVE). A mixture of 4-chlororesorcinol (1 g, 6.9 mmol), tricarballylic acid (1.2 g, 6.9 mmol) and methanesulfonic acid (10 mL) were heated to 80°C for 1 h. The red mixture was poured onto ice and the red-black precipitate was filtered and air-dried. The solid was dissolved in Hepes buffer (0.1 M, pH 7) and extracted with ethyl acetate. The organic phase was discarded, and the aqueous phase was acidified with conc HCl. The resulting slurry was extracted with ethyl acetate, dried ($MgSO_4$) and concentrated to a red solid (0.24 g).

Dye succinimidyl esters. The succinimidyl esters of 5-FAM, 6-JOE, 6-TMR and 6-ROX are commercially available from ABI. All of the remaining dyes were activated using dicyclohexylcarbodiimide and *N*-hydroxysuccinimide in ethyl acetate. A representative example is given below for the synthesis of NAN succinimidyl ester.

NAN succinimidyl ester. 5- (and 6-) carboxy-4,7-dichloro-1',2',7',8'-dibenzofluorescein (NAN, 2 mg, 4 mmol) was suspended in ethyl acetate. Dilute HCl was added and the dye (presumably the lactone form) became completely soluble in the organic phase. The layers were separated and the organic layer dried ($MgSO_4$) and filtered. To the dye solution was added *N*-hydroxysuccinimide (10 mg, 90 mmol) and dicyclohexylcarbodiimide (2 mg, 10 mmol). The reaction progress was monitored by thin-layer chromatography (silica gel, 600:60:16 dichloromethane: methanol:acetic acid). The succinimidyl esters had much higher R_f values (0.5, 0.6) than the free acids (0.1). In addition, the 5- and 6-carboxy isomers were separable by column chromatography under these solvent conditions.

Dye-labeled terminators. 2',3'-Dideoxynucleotide triphosphates with 3-amino-1-propyn-1-yl linker arms were kindly provided by John Van Camp at ABI and were prepared by methods described by Prober, *et. al.*[16] Rhodamine dye terminators were synthesized by John Van Camp by methods described elsewhere[9]. Fluorescein dye-terminators were prepared by methods essentially the same as described previously[16], with the modifications of a higher pH for the coupling reaction (aqueous solution, pH 9.5, instead of DMF/H_2O at pH 7) and the use of HPLC for the purification of the product dye-terminator. In general, the purification required two steps: an anion exchange column (ABI AX300: 40% acetonitrile (ACN)/ 60% triethylammonium bicarbonate (TEAB, 0.1 M), 20 min gradient to 40% acetonitrile/60% triethylammonium bicarbonate (1.2 M)) to separate the free dye, the unlabeled terminator and the labeled terminator. A subsequent, reverse-phase column (ABI RP300: 100% triethylammonium acetate (0.1 M), 20 min gradient to 75% TEAA (0.1 M)/25% ACN) was used to separate the 5- and 6-carboxy isomers of the dye-labeled terminators.

Sequencing reactions (fluorescein terminators). Annealing reaction: Five-fold concentrated sequencing buffer (5× SB) contained MOPS (200 mM, pH 7.5), NaCl (250 mM), $MgCl_2$ (50 mM), $MnCl_2$ (25 mM) and sodium isocitrate (75 mM). The annealing and extension reactions and subsequent ethanol precipitation were performed in one microcentrifuge tube (1.5 mL) for each template. The annealing mixture (6−12 μL) contained 2 μg of template DNA (0.8 pmol for M13mp18), universal or custom primer (1.6 pmol), and 5× SB (4 μL). The solution was incubated at 65°C for five minutes, then slowly cooled to room temperature over 20 min.

Extension reaction. To the annealed reaction mixture was added 4 μL of 2 mM dNTPαS (final concentration of 400 μM of each dNTPαS), and 4 μL of dye-labeled terminator solution [final concentrations are indicated for: ddA-LOU2 (1.2 μM), ddC-5ZOE (1.8 μM), ddG-5NAN (3.2 μM), ddT-6FAM (0.44 μM)]. T7 DNA polymerase (13 U) and inorganic pyrophosphatase (0.012 U) were added to the mixed solution. The final volume was adjusted to 20 μL. (In some cases, the concentrations of dNTPαS, terminators, and enzymes were reduced by factors of four, four and 32, respectively, without noticeable effect.) The extension reaction was incubated at 37°C for 10 min. To the reaction mixture was added a solution of NH_4OAc (9.5 M, 20 μL) and ethanol (95%, 90 μL). The DNA was pelleted by centrifugation (12,000×g) for 15 min and the contents decanted. The precipitated DNA was washed twice with 70% ethanol (300 μL), each wash consisting of brief mixing, a five-minute spin and removal of the wash solution. The precipitated DNA was dried in a vacuum centrifuge for 5−10 min.

Gel analysis using the ABI Model 373. The dried extension products were dissolved in a 5:1 solution of deionized formamide and 50 mM EDTA (4 μL). The microcentrifuge tube was heated to 95°C for two min to denature the DNA. The formamide solution was loaded onto a lane of the sequencing gel (8 M urea, 6% polyacrylamide) in a 373 DNA sequencer. The 373 had been modified to include a filter wheel with five bandpass filters, each of ten nm spectral bandwidth centered at 530, 545, 560, 580, and 610 nm. A modified instrumental software was written to spin the wheel so that one of two combinations could be chosen: 530, 560, 580, and 610, or 530, 545, 560, and 580. (The former combination is compatible with the commercially available fluorescent dye chemistry from ABI; the latter combination is optimized for the T7 DNA polymerase terminators.) Electrophoresis was conducted at constant power (35 W) for 14 h.

Sequencing reactions (rhodamine terminators). Reactions using the four rhodamine terminators, which were developed for taq DNA polymerase, were performed essentially as described above. The annealing mixture (4 μL) contained 1 μg DNA, primer (0.8 pmol), and buffer. The buffer consisted of Tris-HCl (final concentration in extension reaction of 20 mM, NaCl (50 mM), $MnCl_2$ (2 mM), and isocitrate (15 mM). The final concentration of dNTPαS was 200 μM each of dATPαS, dCTPαS, dITPαS, and dTTPαS. The concentrations of dye-labeled terminators

were: ddA-5-carboxyrhodamine 6G (0.1 μM), ddC-6-carboxyrhodamine X (1.2 μM), ddG-5-carboxyrhodamine 110 (0.01 μM), and ddT-6-carboxytetramethylrhodamine (0.7 μM). The quantity of enzymes added were also slightly different: T7 DNA polymerase (2.5 U) and inorganic pyrophosphatase (1 U, from Sigma). The ethanol precipitation was performed with NaOAc added as the additional source of counter-ions.

Sequencing reactions (dye primers). Reactions using c^7dGTP were performed as described in the instructions in the Sequenase® Dye-primer Sequencing kit. Reactions using dNTPαS's were performed in the same manner with the substitution of twice the conc of dNTPαS's for dNTPs. Each primer was annealed to single-stranded M13mp18 DNA in a solution (4 μL for A and C, 11 μL for G and T) containing dye primer (0.4 pmol for A and C, 0.8 pmol for G and T), M13mp18 (0.25 μg for A and C, 0.75 μg for G and T) and 5× SB (1 μL for A and C, 3 μL for G and T). The mixture was heated to 65°C for 5 min and slowly cooled to RT over 20 min. Each extension-termination mixture (6 μL for A and C, 15 μL for G and T) contained dNTP solution [either dNTPs with c^7dGTP (170 μM) or dNTPαS's (340 μM)], and the appropriate ddNTP (0.55 μM). T7 DNA polymerase and pyrophosphatase (2 U/0.002 U for A and C, 4 U/0.004 U for G and T) were added and the reactions incubated for 10 min. Stop solution (15 μL of 20 mM EDTA, 1 M NaOAc, pH 8) was added to the T reaction and the contents of the A, C, and G tubes were added to the T tube. Ethanol precipitation was performed as described, above.

Dye terminator assay. Annealing reactions were performed as described in the sequencing reactions for the fluorescein terminators. Extensions reactions were performed with T7 DNA polymerase (13 U) and pyrophosphatase (0.012 U), dNTPαS (final concentration of 400 μM) and a single dye-labeled terminator: ddA-dye (6 μM), ddC-dye (4−8 μM), ddG-dye (8 μM), or ddT-dye (2−4 μM). Ethanol precipitations were performed using NH_4OAc as described above.

Processivity assay. A solution (144 μL) containing 5× SB (48 μL), M13mp18 (12 μg = 4.8 pmol), 5-carboxyfluorescein (FAM) labeled −21M13 primer (10 pmol) was annealed as described, above. The annealed primer-template was divided into two tubes. To one tube was added four dNTPαS's to a final concentration of 400 μM; to the other tube was added four dNTPs to a final concentration of 200 μM. The volume of each tube was adjusted to 60 μL. Each tube was incubated at 37°C for two minutes. Reactions were initiated by the addition of T7 DNA polymerase (13 U) and pyrophosphatase (0.012 U). Aliquots (6 μL) were removed at various time points (t=0, 0.5', 1', 2', 3', 4', 8' and 12') and the extension reaction was stopped with a solution (12 μL) containing blue dextran (0.75%), NaOH (45 mM), glycerol (7.5%) and EDTA (12 mM). A portion (6 μL) of the reaction products at each time point was analyzed by denaturing (30 mM NaOH) agarose gel electrophoresis on the ABI model 362 GENESCANNER. The experiment was repeated using three-fold the amount of T7 DNA polymerase and pyrophosphatase (39 U and 0.036 U, respectively).

Generation of A-ladders with varying ratios of ddA/dNTPαS. A solution (44 μL) containing 5× SB (22 μL), M13mp18 (11 mg = 4.4 pmol) M13mp18 and FAM-labeled −21 primer (8.8 pmol) was annealed as described above. The solution was divided into aliquots of 4 μL into 1.5 mL microcentrifuge tubes. To each tube was added four dNTPαS's (final conc of 400 μM) and varying amounts of ddATP (final conc of 1 to 25 μM). The tubes were preheated at 37°C for two minutes. Each extension reaction was initiated by the addition of T7 DNA polymerase (4.3 U) and pyrophosphatase (0.004 U). After 10 min at 37°C, the reactions were stopped with EDTA (250 mM, 1 μL). An aliquot (3 μL) was removed from each extension reaction, mixed with formamide (3 μL) and loaded onto a sequencing gel on the ABI model 373 DNA sequencer.

PCR, T7 gene 6 exonuclease and sequencing. PCR Amplification. Ten-fold concentrated PCR buffer (10× PCR buffer) contained 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 0.1% (w/v) gelatin. The dNTP mix contained 1.25 mM each of dATP, dCTP, dGTP and dTTP in Tris-HCl (10 mM, pH 8.0) and EDTA (0.1 mM). PCR was performed with bacteriophage lysate, DNA (0.01−1.0 mg), or bacterial colonies or bacteriophage plaques which were transferred to a small amount of water or TE buffer. Each template DNA which was to be sequenced with the fluorescein terminators required two PCR reactions to generate adequate template for sequencing. Each PCR reaction contained 10× PCR buffer (5 μL), dNTPs (8 μL), −40 primer (2.5 μL of 20 μM), reverse primer (2.5 μL of 20 μM), template DNA and taq DNA polymerase (1 U). The total volume was adjusted to 50 μL.

Thermal cycling was performed on either a Perkin-Elmer Cetus thermal cycler, or a Perkin-Elmer Cetus Model 9600 thermal cycler. If the former was used, each reaction was overlaid with light mineral oil (80 μL) and preheated (95°C) for 2 min. The subsequent thermal cycle (94°C for 30 s, 55°C for 30 s, 72°C for 60 s) was performed for 35 cycles. If the 9600 instrument was used, the oil was omitted and the thermal cycle (92°C for 10 s, 55°C for 60 s, 72°C for 60 s) was performed for 35 cycles.

Reaction with T7 Gene 6 exonuclease. Ten-fold concentrated T7 gene 6 exo buffer (10× exo buffer) contained Tris-HCl (500 mM,

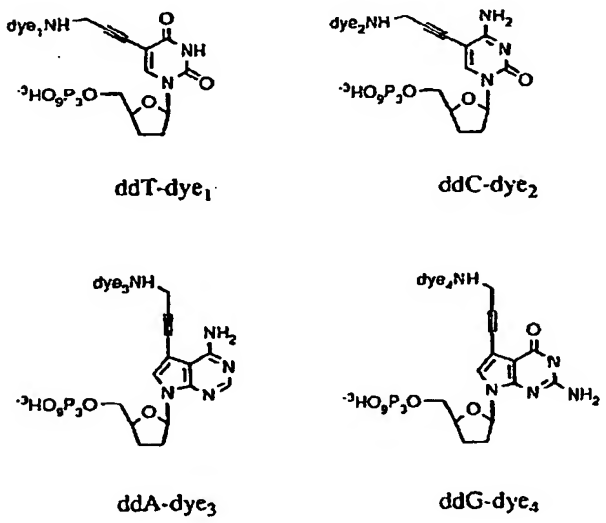

Fig. 1. Structures of the nucleotide portion of the dye-labeled terminators. The syntheses were originally designed and performed by Prober, et. al.[5]

pH 8.1), $MgCl_2$ (50 mM), KCl (200 mM), and 2-mercaptoethanol (50 mM). Each PCR reaction was transferred to a 1.5 mL microcentrifuge tube. To the tube was added T7 gene 6 exonuclease (120 U), 10 exo buffer (10 μL) and water, to a final volume of 100 μL. After incubation at 37°C for 30 min, a solution of phenol:chloroform (100 μL, 1:1) was added to each tube, the mixture was mixed well and centrifuged (13,000×g) for five min. The aqueous phases were transferred to a 1.5 mL microcentrifuge tube, and extracted once with water-saturated ether or chloroform.

Purification by PEG precipitation. PEG solution contained PEG-8000 (40% w/v) and $MgCl_2$ (10 mM). Two PCR samples were combined. To each combined sample was added NaOAc (16 μL, 3M, pH 4.8) and PEG solution (40 μL). The samples were mixed, incubated at RT for 10 min, and the DNA was pelleted by centrifugation (10,000×g) for 15 min at RT. All the supernatant was removed by aspiration. The DNA pellets were washed twice with 100% ethanol and dried briefly under vacuum. The DNA was dissolved in 7.5 μL of water. Each sequencing reaction required 6.5 μL of the DNA solution and utilized −21 primer.

RESULTS AND DISCUSSION

Dye Terminator Composition. Fig. 1 shows the structures of the nucleotide portion of the terminators. Initial experiments focused on using T7 DNA polymerase with the rhodamine-labeled set of dye terminators, which were developed for taq DNA polymerase, to examine the pattern of termination. Many variations in the composition of polymerase substrates and cofactors were tested, including the use of Mg^{2+} and Mn^{2+}, and the use of analogs of dNTPs. The best results with the rhodamine terminators, shown in Fig. 2, *panel a*, utilized Mn^{2+} and four

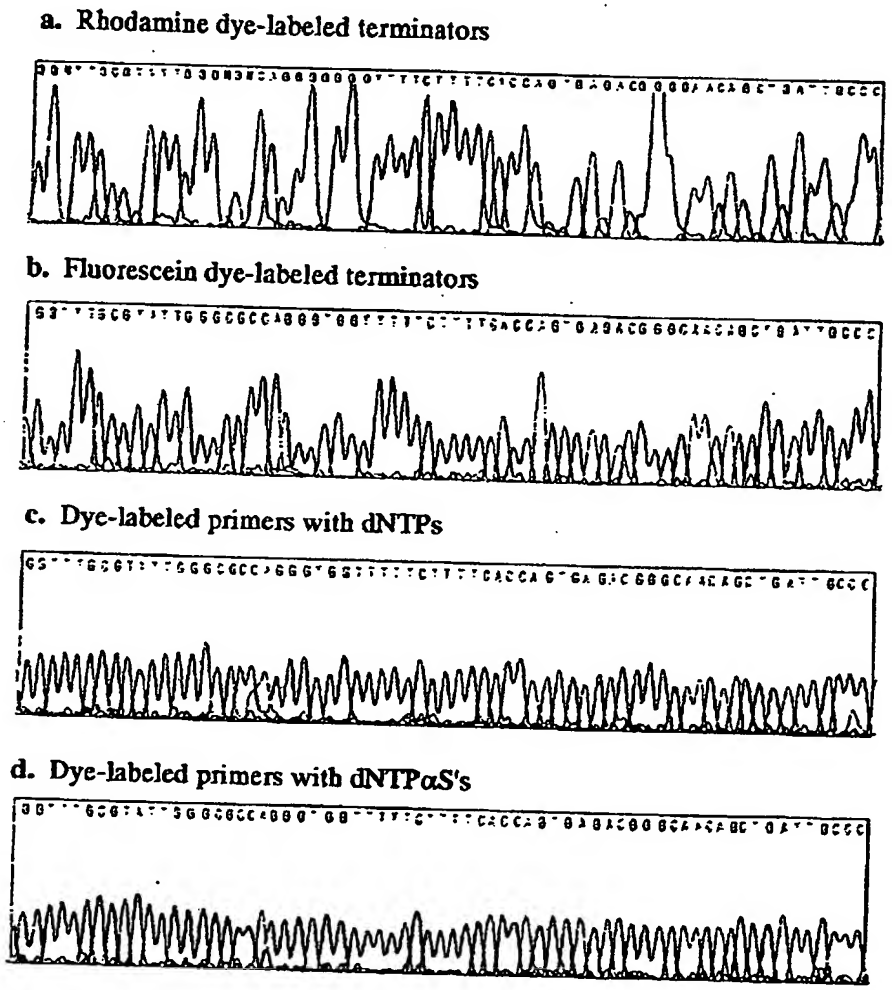

Fig. 2. Effect of different dyes and dNTPs on four-color sequencing with T7 DNA polymerase and Mn^{2+}. Single-stranded M13mp18 (1−2 μg) was annealed with −21 primer. The sequencing method utilized dye-labeled terminators (*panels a* and *b*) or dye-labeled primers (*panels c* and *d*). The dNTP solution contained dATPαS, dCTPαS, dTTPαS and dTTPαS (*panel a*); four dNTPαS's (*panels b* and *d*); or four dNTPs (*panel c*).

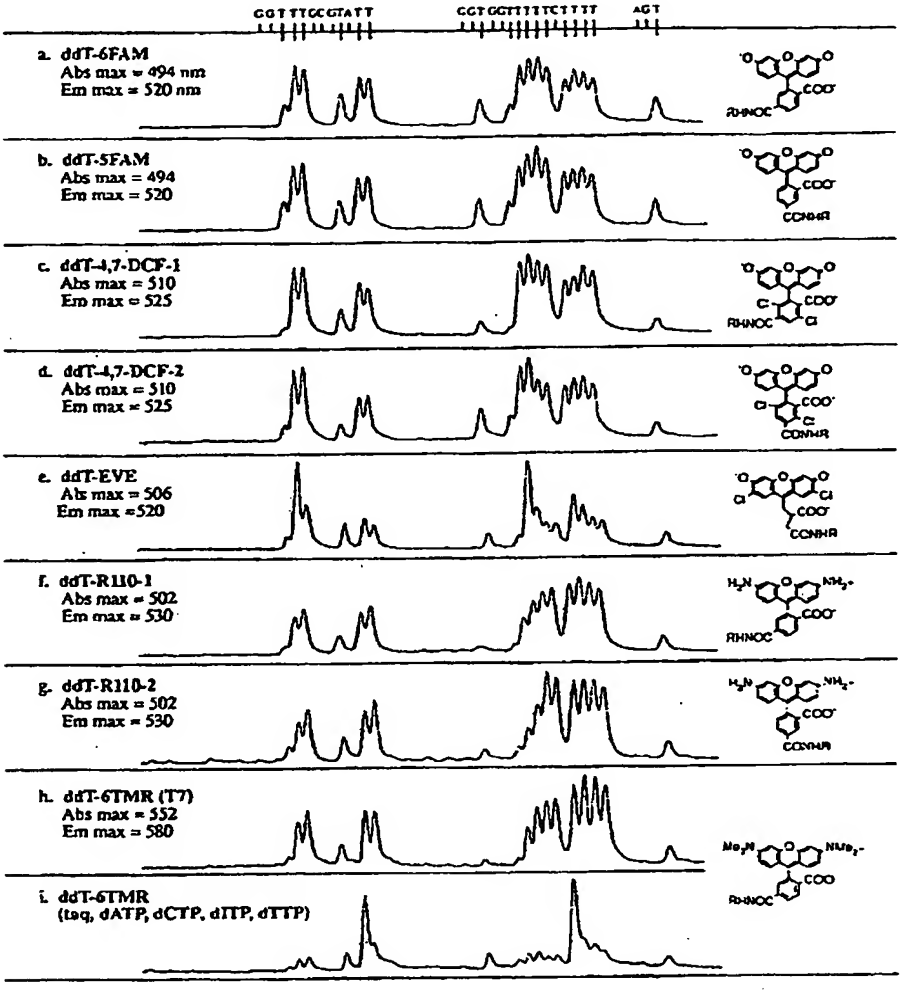

Fig. 3. Termination patterns of different dye-labeled terminators (ddT-dye). The structure of the nucleotide (R) is shown in Fig. 1. Each extension reaction contained −21 primer annealed to ss M13mp18, Mn^{2+}, Mg^{2+}, four dNTPαS's, T7 DNA polymerase and pyrophosphatase.

2'-deoxyribonucleoside 5'-*O*-(1-thiotriphosphates) (dNTPαS's): dATPαS, dTTPαS, dCTPαS, and dITPαS. The use of dITPαS alleviated the compression caused by the rhodamine terminators. For any of the dye terminators described in this paper, the use of four dNTPαS's generally gave more even patterns of termination with T7 DNA polymerase for dye-labeled ddCTP and dye-labeled ddTTP, and slightly less even patterns for dye-labeled ddGTP and dye-labeled ddATP. Use of analogs of dGTP, such as 7-deaza-2'-deoxyguanosine 5'-triphosphate (c^7dGTP) or 2'-deoxyinosine 5'-triphosphate (dITP), gave less even patterns of termination.

The termination pattern was quite acceptable for the rhodamine dyes (Fig. 2, *panel a)*, except for the sequences in which T or C follow G. In these sequences almost no signal is visible. Interestingly, the same sequences which gave the smallest peaks with T7 DNA polymerase also gave the smallest peaks when taq DNA polymerase was used with the rhodamine dye terminators.

The next step towards improving the pattern of termination was to change the type of dye. We investigated analogs of fluorescein dyes since many spectrally resolvable analogs were available. Negatively charged fluorescein dyes have several advantages over zwitterionic rhodamine dyes. Fluorescein dyes on terminators do not cause additional compressions in the gel electrophoresis and therefore do not require the use of dITP. The fluorescein terminators have a net −5 charge and migrate through the gel before the DNA sequence bands, while rhodamine terminators, with a net −3 charge, migrate during the sequence and must be removed thoroughly from the reaction mixture before loading onto the gel. Finally, fluorescein terminators produce a better pattern of termination with T7 DNA polymerase, as shown in Figs. 3–6.

Since dye-labeled ddTTP and ddCTP generally gave the least uniform pattern of the terminators, several dyes were tested on both nucleotides. In general, within a class of dyes, the smaller

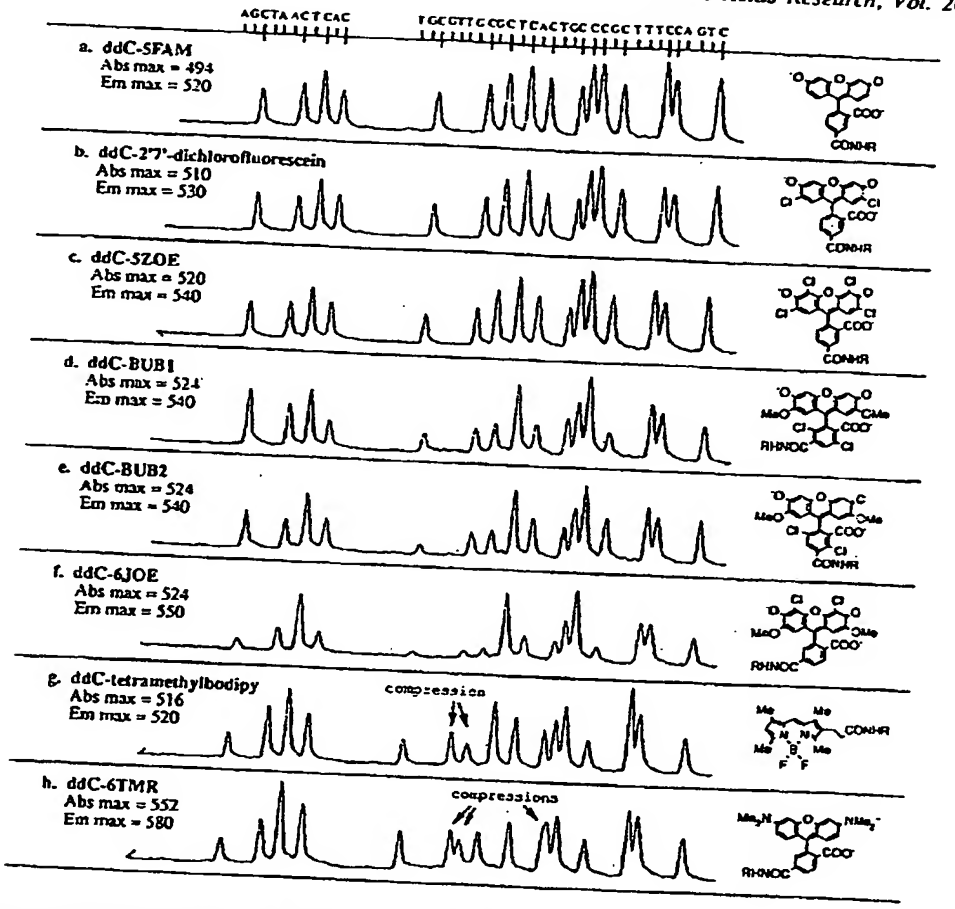

Fig. 4. Termination patterns of different dye-labeled terminators (ddC-dye). The structure of the nucleotide (R) is shown in Fig. 1. Each extension reaction contained −21 primer annealed to ss M13mp18, Mn^{2+}, Mg^{2+}, four dNTPαS's, T7 DNA polymerase and pyrophosphatase. The compressions for the zwitterionic dyes (*panels G* and *H*) are resolved when dITP or dITPαS is substituted for dGTPαS.

the dye, the better. In an attempt to find four spectrally resolvable dyes, however, variations on the dye structure were necessary to shift the emission maxima of the dyes to longer wavelengths. Figs. 3 and 4 show that T7 DNA polymerase only gave acceptable patterns with ddT-dye and ddC-dye when small dyes were used. The longer wavelength, and therefore, bigger, dyes, such as 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein (JOE) and 5-carboxy-4,7,4',5'-tetrachloro-2',7'-dimethoxyfluorescein (LOU), gave small peaks for Ts and Cs following Gs. The use of small dyes did not guarantee uniform patterns, however. Both bodipy and EVE dyes, which are smaller than the fluorescein analogs, gave relatively uneven termination patterns.

The best set of spectrally resolvable dyes for ddTTP-dye and ddCTP-dye were obtained with ddT-6FAM and ddC-5-carboxy-2',4',5',7'-tetrachlorofluorescein (ddC-5ZOE). With these in hand, we found that almost all the fluorescein dyes tested on ddATP and ddGTP produced acceptable patterns (see Figs. 5 and 6). Even the very large bifluor dye, which is a rhodamine dye attached to a fluorescein dye, produced a fairly even termination pattern on ddG (although it causes band compressions, as well). The two longest wavelength (and structurally most complex) dyes were reserved for the purine terminators. Fig. 2, *panel b* shows an example of single-stranded M13mp18 DNA sequenced using the four fluorescein dye terminators and a set of four bandpass filters which were chosen to spectrally resolve the new dye set.

Use of dNTP Combinations to Resolve Gel Compressions. Although the initial purpose of using dNTPαS's was to improve the pattern of termination, we soon observed that some sequence compression problems which normally required the use of c^7dGTP in radioisotopic or dye primer sequencing were completely resolved using the four dNTPαS's with the fluorescein dye terminators. By sequencing a hairpin region consisting of a 11 base pair stem and a six base loop with the 16 possible combinations of dNTPαS's and dNTPs, we found that dCTPαS was essential in resolving the compression. Furthermore, we observed that the combination of dCTPαS and dGTPαS was the most effective in resolving the compression, regardless of the composition of the remaining deoxynucleotides (Fig. 7). The use of dITPαS with three dNTPs also resolved the compression. For our purposes, the optimum effect of uniform pattern and absence of compression required the use of all four dNTPαS's.

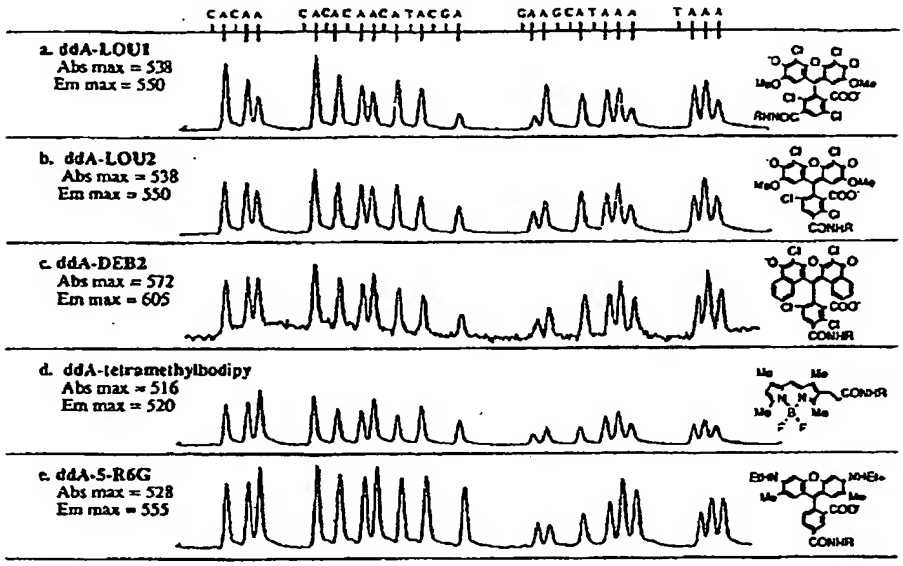

Fig. 5. Termination patterns of different dye-labeled terminators (ddA-dye). The structure of the nucleotide (R) is shown in Fig. 1. Each extension reaction contained −21 primer annealed to ss M13mp18, Mn^{2+}, Mg^{2+}, four dNTPαS's, T7 DNA polymerase and pyrophosphatase.

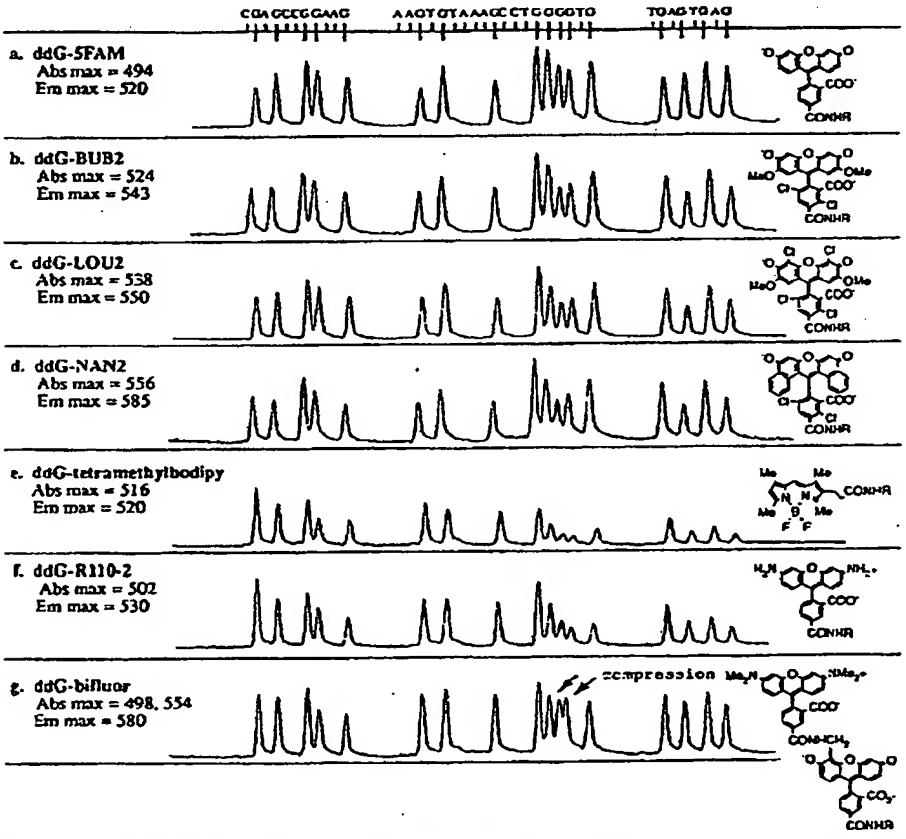

Fig. 6. Termination pattern of different dye-labeled terminators (ddG-dye). The structure of the nucleotide (R) is shown in Fig. 1. Each extension reaction contained −21 primer annealed to ss M13mp18, Mn^{2+}, Mg^{2+}, four dNTPαS's, T7 DNA polymerase and pyrophosphatase.

a. c^7dGTP, dATP, dCTP, dTTP: correct sequence, but uneven pattern

b. dATPαS, dCTPαS, dGTPαS, dTTPαS: correct sequence, good pattern

c. dCTPαS, dGTPαS, dATP, dTTP: correct sequence

d. dCTPαS, dATP, dGTP, dTTP: correct sequence, but compressed

e. dGTPαS, dATP, dCTP, dTTP: incorrect sequence

f. dATP, dCTP, dGTP, dTTP: incorrect sequence

g. (S_p)-dITPαS, dATP, dCTP, dTTP: correct sequence

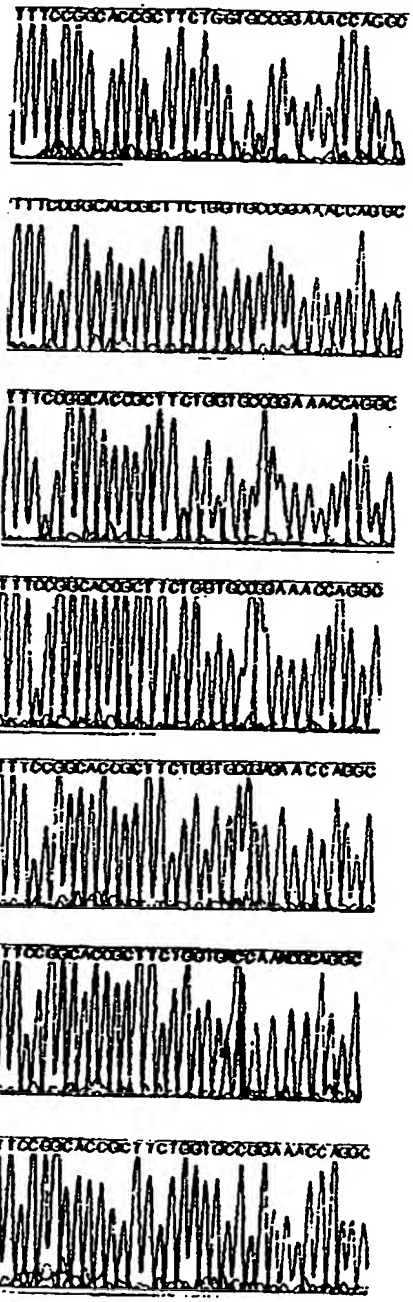

Fig. 7. Effects of different dNTP compositions on the resolution of a hairpin sequence. The sequence consisted of an 11 base pair stem and six base loop (5'-TTTCCGGCACCGCTTCTGGTGCCGGAAA-3'). Each extension reaction contained M13mp18 annealed to a 17-bp primer at base 6571, four fluorescein-labeled terminators, Mn^{2+}, Mg^{2+}, T7 DNA polymerase and pyrophosphatase.

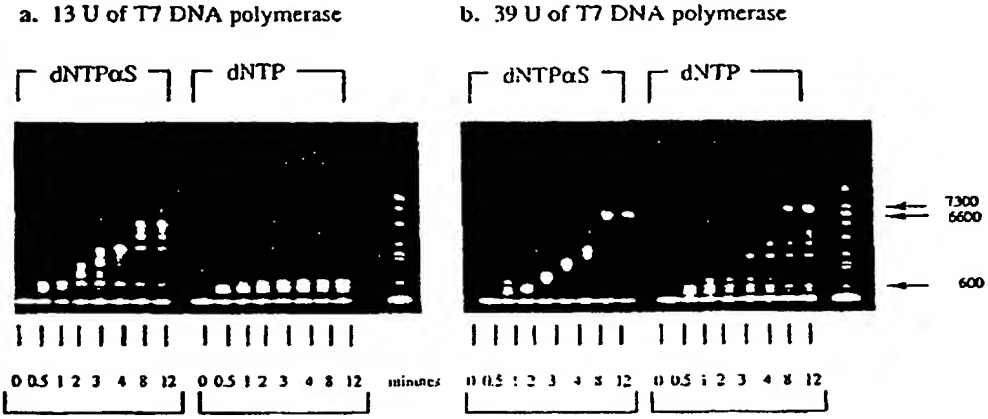

Fig. 8. Assay comparing the activity of T7 DNA polymerase with four dNTPαS's or with four dNTPs. The template was M13mp18 (12 μg) annealed with FAM-labeled −21 primer. The amount of DNA polymerase was 13 U (*panel a*) or 39 U (*panel b*). Aliquots were removed at 0.5, 1, 2, 3, 4, 8, and 12 min and added to stop solution. The extension products were examined on a denaturing (30 mM NaOH) agarose gel on the ABI model 362 GENESCANNER. The molecular weights of three bands in the size standard are indicated.

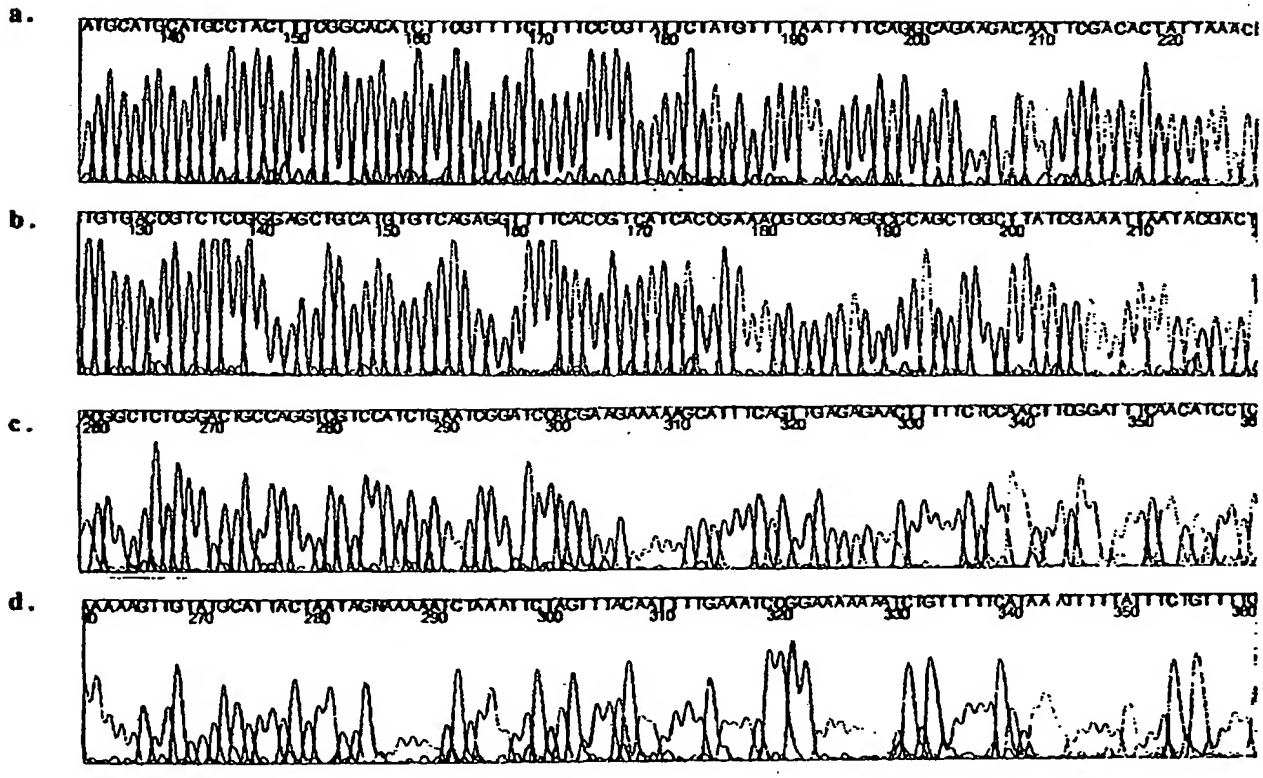

Fig. 9. Representative sequencing data from two PCR products (*panels a* and *b*) and from two single-stranded phagemids (*panels c* and *d*). The PCR samples were 500 and 4,500 base-pairs in length, respectively. Each PCR sample was digested with T7 gene 6 exonuclease, precipitated with PEG and sequenced with fluorescein-labeled terminators. Data is shown from approximately base 130 to 320 from the primer; useful data was obtained to base number 350. The phagemid samples were purified by standard methods[21]. Data is shown from base 260 to 360.

Dye-labeled Primer Sequencing with dNTPαS's. Initial experiments with dye primer sequencing showed that the termination pattern with four dNTPαS's was as uniform as with dNTPs (Fig. 2, *panels c and d*). A few minor compressions which were observed with c^{7}dGTP were entirely resolved when dNTPαS's were used.

Processivity of T7 DNA Polymerase with dNTPαS's. A qualitative idea of how T7 DNA polymerase behaves when all four dNTPs are substituted with dNTPαS's was obtained in a method analogous to that of Tabor and Richardson[10]. A fluorescent dye-labeled primer was annealed to single stranded M13mp18 DNA and chain elongation was initiated by the addition of T7 DNA polymerase, pyrophosphatase and either four dNTPαS's or four dNTPs. Aliquots of the reaction mixture were removed at various time points, the enzyme activity stopped with EDTA and the products analyzed by denaturing agarose gel electrophoresis on the ABI Model 362 GENESCANNER (Fig. 8). The extension reaction with dNTPαS's showed a very different profile than when dNTPs were incorporated. At the lower concentration of T7 DNA polymerase, the enzyme seemed to 'pause' at about base 600 when dNTPs are used. With dNTPαS's, the enzyme synthesized longer extension products, even at the lower enzyme concentration. At higher enzyme concentrations, reactions with either of the dNTP solutions proceeded more efficiently, although the dNTPαS extensions left fewer pauses. These results can be interpreted as higher enzyme efficiency with Mn^{2+} when dNTPαS's are used.

Sequencing of PCR Products with Gene 6 Exonuclease Digestion. PCR products are effectively sequenced by cycle sequencing[17], or by digestion with T7 gene 6 exonuclease[18] to provide single-stranded template. We show here two examples of PCR products sequenced by the latter method (Fig. 9). The PCR products were treated with T7 gene 6 exonuclease (120 U) for 30 min followed by a phenol/chloroform protein precipitation. The reaction products were purified by PEG precipitation before sequencing.

Probability Analysis of Termination Products. The amount of dideoxynucleotide and deoxynucleotides in Sanger sequencing are generally adjusted by trial and error so that a reasonable amount of signal from the beginning to end (balance) is obtained. However, Tabor and Richardson's work with T7 DNA polymerase and Mn^{2+}, which showed almost perfectly uniform peak sizes, suggested that peak sizes and balance could be described mathematically[12]. By making an initial assumption about the probability of termination, we have derived equations which relate the probability of termination to the size of the n^{th} peak. Four-color, one-pot dye-terminator reactions can also be described, and the balance for each color can be adjusted independently.

Analysis of a Repeating Sequence: Total Signal and Balance. Consider a repeating sequence, ACGTACGT.... This sequence is representative of a typical random sequence. Assume that the probability of termination at a ($p_a \leq 1$), the probability of termination at C is p_c, etc. We can write Equation 1, where R_N is an enzyme-dependent discrimination constant that describes the extent of discrimination of the enzyme between each ddNTP and dNTP pair. For T7 DNA polymerase, which does not discriminate against dideoxynucleotides when Mn^{2+} is used, the value of R is unity for each ddNTP/dNTP pair ($R_A = R_C = R_G = R_T = 1$), and is independent of sequence. The value of R is also unity for each ddNTP/(S_p)-dNTPαS pair. Taq polymerase, by contrast, discriminates much more against dideoxynucleotides, and the value of R_A for the unlabeled nucleotide pair, ddATP/dATP, is 0.0003 (based on the ratio of ddATP/dATP used in sequencing[19]). For taq, the values of R vary for each ddNTP/dNTP pair and is dependent on local sequence.

$$p_n = R_N \frac{[\text{ddNTP}]}{[\text{dNTP}]} \qquad (1)$$

Assume that the total amount of primed template is D_o. The amount of terminated primer in the first band is:

$$d_1 = p_a D_o$$

That is, the molar amount of DNA in the first peak is the product of the probability of termination and the initial amount of primed template. The molar amount of 3′ hydroxyl on the extended primer which is available to continue polymerization is reduced by the amount consumed in the first peak. Therefore, the molar amount of the next peaks are:

$d_{1a} = p_a D_o$ (d_{1a} designates the first A peak)
$d_{1c} = p_c D_o (1-p_a)$ (d_{1c} designates the first C peak)
$d_{1g} = p_g D_o (1-p_a)(1-p_c)$
$d_{1t} = p_t D_o (1-p_a)(1-p_c)(1-p_g)$
$d_{2a} = p_a D_o (1-p_a)(1-p_c)(1-p_g)(1-p_t)$

We can define the probability of not terminating at the ACGT four-base sequence as $(1-p)$. Therefore, we can write:

$$1-p = (1-p_a)(1-p_c)(1-p_g)(1-p_t) \qquad (2)$$

Using Equation 2, we can continue the series to arrive at an equation which describes the size of the n^{th} A peak in either the dye-primer or dye-terminator system:

$$\begin{aligned} d_{2a} &= p_a D_o (1-p) \\ d_{3a} &= p_a D_o (1-p)^2 \\ d_{na} &= p_a D_o (1-p)^{n-1} \end{aligned} \qquad (3)$$

For example, take the case of an A reaction using traditional primer-labeled sequencing in our repeating four-base sequence. Since there is no ddCTP, ddGTP or ddTTP in the reaction, we can set p_c, p_g, and p_t to zero. Substituting these values into Equation 2, Equation 3 can be written as the simpler form of Equation 4. In order to maximize the size of the n^{th} peak relative to the n^{th} peak for different termination probabilities, the first derivative of Equation 4 is set to zero, as shown in Equation 5.

$$d_{na} = p_a D_o (1-p_a)^{n-1} \qquad (4)$$

$$0 = \frac{(d_{na})}{d(p_a)} = -p_a D_0 (n-1)(1-p_a)^{n-2} + D_0 (1-p_a)^{n-1};\ p_a = \frac{1}{n} \qquad (5)$$

To maximize the size of the 500th peak, or 125th A peak in our repeating sequence, the probability of termination (p_a) is set to 1/125. For T7 DNA polymerase the enzyme discrimination constant, R, is unity for each ddNTP/dNTP or ddNTP/(S_p)-dNTP pair, so the ratio of each ddNTP/dNTP should be 1/125, or 0.008.

For the dye terminator system, the enzyme constants vary by a factor of approximately three or four, depending on the local sequence. In general, the discrimination constants are smaller for the dye-labeled nucleotides following a series of Gs. The values for each constant, R, were estimated by testing a range of concentrations of each terminator with a fixed concentration

Table 1. Ratios of ddNTP-dye/dNTPαS in single- and four-color reactions; values of enzyme discrimination constants (R_B) for dye-terminators

Dye Terminator	Single-color reactions: ratio of ddNTP-dye/(R_p-S_p)-dNTPαS (μM/μM)	R_B	Four-color reactions: p_a	ratio of ddNTP-dye/(R_p-S_p)-dNTPαS (μM/μM)
ddA-LOU2	6/400	0.3	0.0016	1/400
ddC-5ZOE	9/400	0.2	0.0016	2/400
ddG-NAN2	8/400	0.2	0.0032	3/400
ddT-6FAM	2/400	0.8	0.0016	0.4/400

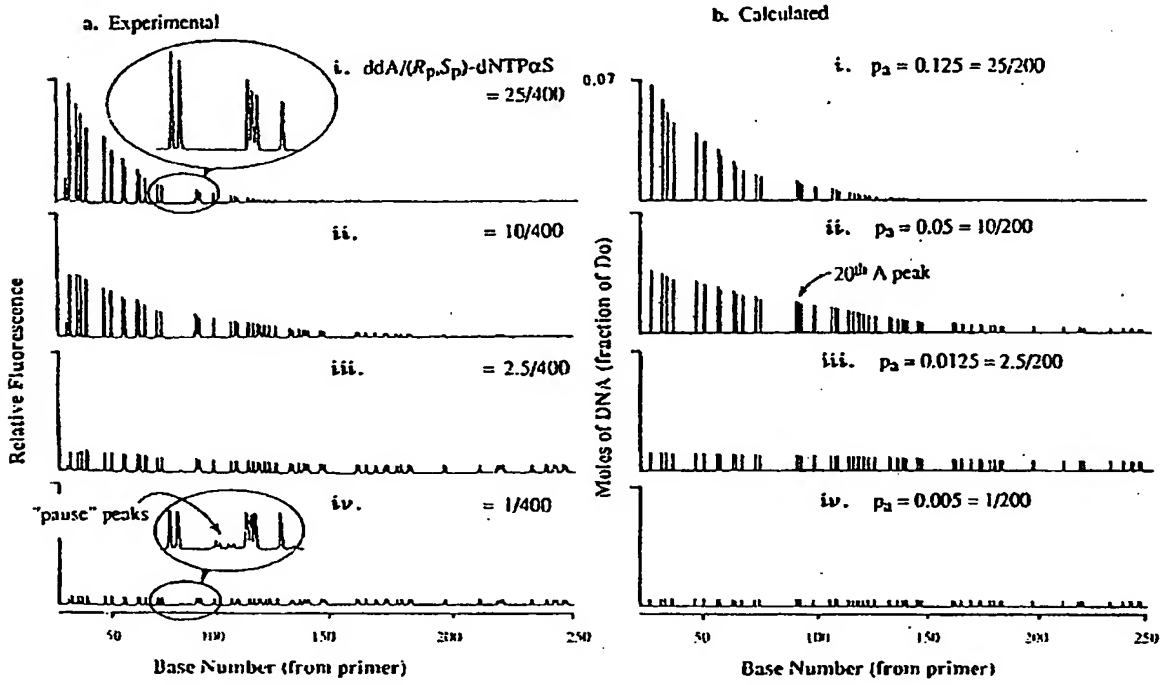

Fig. 10. Comparison of experimental (*panel a*) and calculated (*panel b*) termination patterns with variable ratios of ddATP/dNTPαS. The experimental extension reactions contained M13mp18, FAM-labeled −21 primer, Mn^{2+}, Mg^{2+}, T7 DNA polymerase, pyrophosphatase and ratios of ddATP/(R_p-S_p)-dNTPαS varying from 25 μM/400 μM to 1 μM/400mM. (The ratios of ddATP to the *active* isomers of the dNTPαS's were from 25 μM/200 μM to 1 μM/200 μM.) The fluorescence patterns were collected through the 580 nm filter; all the peaks are shown on the same scale. The first peak in each panel is the 5th A peak following the primer. The calculated termination patterns were obtained by entering the M13mp18 sequence, varying the termination probabilities of A-termination from 0.125 to 0.005 and setting the probabilities of terminating in C, G and T to zero. The linear regression analysis of each fluorescent peak vs. its corresponding calculated molar quantity gave correlation coefficients for *panels i–iv*, of 0.995, 0.989, 0.969, and 0.969.

of dNTPαS in a single color extension reaction (i.e., for the ddA-dye terminator, $p_c = p_g = p_t = 0$, $p = p_a$ in Equation 2). The ratio of ddNTP/dNTPαS which produced the biggest peak at base 500 was chosen as 'optimal'. The 'optimal' ratio of dd/dNTPαS and a value of p_a of 0.008 were used in Equation 1 to arrive at values for R given in Table 1.

In determining the ratios of the eight reagents (four dNTPs, four dye-labeled ddNTPs) in a one-pot extension reaction, the brightness of the dyes must also be considered. Although all the dyes have good quantum yields ($\Phi > 0.5$), the longer wavelength dyes are not excited near their absorbance maxima by the argon ion laser and are dimmer than the shorter wavelength dyes. Best results in four-color sequencing are obtained when the values for the termination probabilities (p_a, p_c, p_g, p_t) are set higher for those terminators with dim dyes, and lower for the brighter dyes.

Using Equation 3, we can see that the size of the n^{th} peak depends on the probability of not terminating at n bases (1-p). When p_a, p_c, p_g and p_t in Equation 2 are small, the higher order terms can be ignored, and:

$$p \cong p_a + p_c + p_g + p_t \qquad (6)$$

That is, we can vary the values of p_a, p_c, p_g and p_t independently without changing the size of the n^{th} peak as long as their sum is a small constant, p.

With the T7 DNA polymerase terminators, the dyes on A, C and T are approximately equivalent in brightness, but the dye

on G is about half as bright. Therefore, we can arrange the ratios in Equation 6 so that the probability of termination in G is twice that of any other base, and so the sum of the termination probabilities is 0.008 ($p_a + p_c + p_g + p_t = p = 0.008$). Table 1 gives the ratios for single and four color reactions required to maximize the 500th nucleotide peak and the estimated enzyme discrimination constants, R.

The pattern of termination for a specific sequence can be calculated from the sequence, the ratio of ddNTP/dNTP, and the termination probabilities. We have written a software program which requests sequence (e.g., AGCCAA), termination probabilities (p_a, p_c, p_g, p_t) and amount of template (D_o). The program calculates the quantities of DNA in each peak (d_n) based on Equation 3. The data can be displayed in a spreadsheet and plotted in a bar graph format to generate the familiar sequencing pattern. Fig. 10, *panel b* shows the results of a calculation to generate the A termination pattern with M13mp18 for different values of p_a. The M13 sequence was entered, the probabilities of termination in C, G and T were set to zero (p_c, p_g, $p_t = 0$), and the probability of termination in A was varied from 0.125 to 0.005. Fig. 10, *panel a* shows the results of a sequencing experiment using single-stranded M13mp18 DNA, dye-labeled primer, four dNTPαS's, T7 DNA polymerase, and varying concentrations of ddATP. T7 DNA polymerase and dye primers were used since the enzyme discrimination constants are independent of sequence and best fit the model. The ratios of ddATP/dNTPαS were varied to give the same termination probabilities as in Fig. 10, *panel β*, calculated using Equation 1, where $R_A = 1$, and where the concentration of dNTPαS is divided by two, since only half of the dNTPαS's are the active (S_p)-diasteriomers[19].

The experimental and calculated patterns correlate well. The correlation coefficients, given in Fig. 10, are the results of a comparison between the calculated molar quantities and the actual fluorescence of each peak (measured in peak height). The 'pause' peaks, or nonspecific terminations, become increasingly apparent at lower values of p_a (Fig. 10, *panel a*: *i* vs. *iv*). The probability of pausing can also be assigned a value (p_p), and the size of the pause peak is the product of p_p and the amount of available primed template. So, if the pause occurs at position n, the size of the pause peak would be $p_p D_o (1-p_a)^{n-1}$. For a given extension incubation time, the pause peaks should increase for decreasing values of p_a.

Each termination probability (p_a) maximizes one peak relative to the corresponding peak for different termination probabilities. For example, in Fig. 10, *panel b.ii.*, the value of p_a is 0.05 or 1/20. Using Equation 5, we can see that the 20th A peak is maximized relative to the 20th A peak in *panels b.i.* or *b.iii.*

CONCLUSIONS

Dideoxy terminators labeled with analogs of fluorescein provide acceptable quality DNA sequencing data on single-stranded DNA with the ABI model 373 DNA sequencer. The main advantages of this method are the simplicity in performing the extension reactions and the ability to use any unlabeled primer. The main disadvantage is the need for fairly high quantities of template DNA (0.8 pmol) as compared to the amount needed for cycle sequencing to achieve adequate signal-to-noise for the automated base-calling sofiware. The termination patterns for this set of terminators is more uniform than either the Du Pont terminators or the ABI taq terminators. The uniformity translates to better signal-to-noise in the sequencing data for adequate signal. The lack of thermal stability of T7 DNA polymerase, however, makes the simple cycling protocols which are so convenient for sequencing double-stranded templates out of reach. This problem may be solved in part by using PCR amplification and T7 gene 6 exonuclease as a means of preparing template DNA. In addition, we are currently working on variations of the alkaline denaturation method[20] as a method to sequence double stranded plasmid DNA using fluorescein terminators.

An additional advantage of this chemistry is that the use of four dNTPαS's instead of c^{7}dGTP with T7 DNA polymerase appears to be a promising method to resolve gel compressions. That dCTPαS appears to be the main component in destabilizing hairpin structures suggests investigation of analogs of dCTP may provide additional nucleotides which are useful for DNA sequencing. In addition, the use of four dNTPαS's with Mn^{2+} appears to improve the efficiency of the enzyme, suggesting that the usefulness of the lesser-used nucleotides (e.g., dITP, c^{7}dITP) may be improved with thiotriphosphate versions.

Modeling the typical DNA sequencing pattern with probability analysis addresses such sequencing issues as maximizing signal, optimizing protocols, and understanding the effects of chemical structures in the extension reactions. Measurement of the termination pattern to arrive at the enzyme discrimination constants may be a simple way to evaluate chain terminators such as AZT or ddI without separately measuring K_m or V_{max} values.

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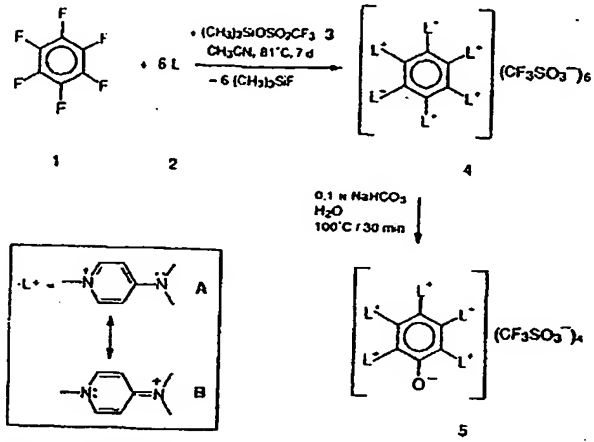
First-Ever Per(onio) Substitution of Benzene: The Role of the Counterion

Robert Weiss,* Bernd Pomrehn, Frank Hampel, and Walter Bauer

In earlier studies we have shown that the transition from neutral to cationic sets of substituents by poly(onio) substitution (POS) with main group element derivatives and nonaromatic organic π systems generally leads to a marked increase in Lewis acidity and electron affinity.[1-6] At the same time, novel reaction pathways are observed for redox and substitution reactions with appropriate reagents.[5, 6] This is attributed primarily to the electrostatic field effect (pole effect)[7] of the cationic substituents. Since electrostatic effects are not, in principle, subject to any saturation effect, but are instead additive in nature, it is of particular interest to introduce the maximum number of onio substituents structurally possible at a basic core unit. In this context, we have now turned our attention to the example of an aromatic π system and report herein on the synthesis, structure, and some properties of the first hexakis(onio)-substituted benzene derivative.[8]

In 1993 Streitwieser et al. reported on an unsuccessful attempt at synthesizing one such hexacationic substituted benzene derivative.[9] Upon reaction of hexafluorobenzene (1) with 4-dimethylaminopyridine (DMAP, 2), an S_NAr sequence gave the corresponding 1,4-bis(onio)-substituted benzene derivative, which was obtained in 20% yield and structurally characterized. An attempt at synthesizing the corresponding per(onio)-substituted salt starting from 1 and DMAP led, after a reaction time of three and a half months, to a higher substituted product in extremely low yield. This was identified on the basis of spectroscopic data as the corresponding pentakis(onio)-substituted benzene. The last fluorine substituent could not, however, be displaced, even under elevated pressure.

The low reactivity of this bis(onio)-substituted and particularly the pentakis(onio)-substituted benzene is at variance with the expectation that the electrophilicity of π systems should be enhanced with an increase in the number of onio substituents. To explain the results of Streitwieser et al., we assume that contacts between the electrophilic countercation and the liberated fluoride ions lead to inhibition of any further nucleophilic substitution. A number of possibilities may be discussed regarding the nature of these contacts, for example the formation of kinetically stable ion clusters or even the formation of stable Meisenheimer complexes (more likely with higher levels of onio substitution). Exchange of fluoride ion for a more strongly nucleofugic anion should then hold the key to successful per(onio) substitution in the benzene system. This simple approach met with complete success and led to the thermodynamically particularly favorable variant of poly(onio) substitution depicted in Scheme 1.



1 2 4 5

Scheme 1. Synthesis and hydrolysis of 4.

The desired per(onio) substitution product 4, the sole reaction product, was obtained in virtually analytically pure form in 92% yield as a sparingly soluble salt. It is clear that the reaction is greatly facilitated by the combination of the S_NAr substitution sequence with the formation of the extremely stable Si–F bond. We do not rule out an assisting intervention by 3 at a relatively early stage of the substitution sequence. The exclusive formation of 4, even upon addition of 2 and 3 in a molar ratio of 1:1:1, underlines most explicitly the electrophilic autoactivation of the π system through increasing onio substitution and confirms our interpretation of the results of Streitwieser et al.[9]

Remarkably, 4 can be recrystallized from hot water without decomposition. This allowed us to obtain colorless needles suitable for X-ray structure analysis,[10] the results of which are depicted in Figure 1 (for clarity four of the triflate ions located at a greater distance from the hexacation are omitted).

The following structural features are noteworthy:

- With a torsion angle of 80.0°, the pyridine rings are almost orthogonal to the benzene ring, whereas the corresponding angle in hexaphenylbenzene amounts to about only 65°.[11] MNDO calculations give an average torsion angle of 83° for

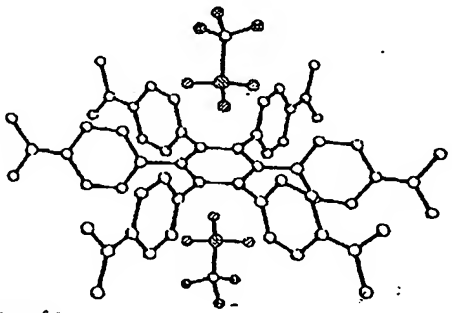


Fig. 1. Crystal structure of 4.

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the cation in 4. The bond lengths in the onio substituents attest to a considerable contribution from resonance structure B (Scheme 1)—a charge-stabilizing effect generally found for onio systems derived from 2.[1, 4, 9, 12]

- Whereas four of the six triflate ions occupy peripheral positions relative to the polycation (in the outer region of the pyridinio substituents), the remaining two triflate ions are located directly above and below the plane of the benzene π system. Thus, the two planes through the oxygen atoms in the triflate groups are almost parallel to that of the benzene ring. Clearly, the hexacation is partially neutralized—in the primary sphere, so to speak—by two electrostatically strongly bound counterions, whereas the remaining four counterions form a distinctly more weakly bound secondary anion sphere. The resulting central triple-decker ion cluster is a completely novel structural element. To the best of our knowledge, this positioning of anions relative to an aromatic π system seen here has not been observed previously. The interplanar distance of 3.2 Å here points to purely ionic interactions. Weak bonding contacts between the oxygen centers and the α-hydrogen atoms of the pyridinio substituents are also evident (mean O—H distance 2.2–2.5 Å).

Cyclic voltammetric investigations of 4 yielded one quasi-reversible reduction at −0.80 V and a second, irreversible one at −1.14 V.[13] This corresponds to an increase in redox potential of 2.55 V relative to that of benzene,[14] which, because of the extensive out-of-plane torsion of the pyridinio substituents, must be essentially electrostatic in origin. The poly(onio)-substituted salts 1,3,5-tris(4-dimethylamino-1-pyridinio)benzene tris(trifluoromethanesulfonate) (6) and 1,2,3,4,5-pentakis(4-dimethylamino-1-pyridinio)benzene pentakis(trifluoromethanesulfonate) (7), which we synthesized in analogy to 4, exhibit (irreversible) $E_{1/2}$ values of −1.34 and −0.88 V, respectively.[13] Figure 2 shows a plot of the redox potentials of the compounds investigated versus the number of pyridinio substituents.

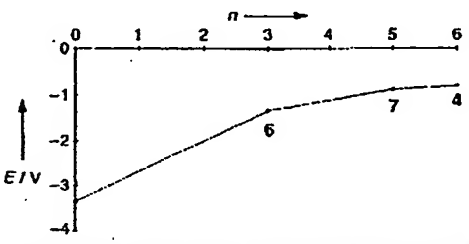

Fig. 2. Dependence of the redox potentials of substituted benzenes on the number n of 4-dimethylaminopyridinio substituents.

It can be seen that the first three onio substituents, arranged alternately, increase the electron affinity of the benzene system much more efficiently than those following. This deviation from the additivity of the substituent effect may be a consequence of the −M effect of the pyridinio substituents, which, due to the conformationally restricted conjugation in 4 and 7, can only be realized for 6. That the redox potential of 4 lies only slightly above that of 7 can be attributed to an increasingly nonlinear contribution from the dimethylamino groups. The importance of the iminium structure B (Scheme 1) increases with increasing electrophilicity of the template such that the positive charge is increasingly shifted to the periphery of the molecule. This hypothesis is supported by the observation that this effect is found only with dimethylaminopyridinio substituents and not with pyridinio substituents.[15, 16]

The ^{13}C NMR spectrum of 4 in solution (CD_3NO_2/CF_3COOD) shows, as expected, only one signal for the benzene ring and one set of signals for the onio substituents. Thus, in solution, (averaged) D_{6h} symmetry can be inferred for the poly(onio) system. In the crystal, on the other hand, 4 is present with a lower degree of symmetry. The asymmetric unit consists of a "half" molecule of 4, with three carbon atoms of the benzene ring, three onio substituents, and three counterions. Consequently, the ^{13}C CP/MAS solid-state NMR spectrum[17] contains three sets of signals for the cation in 4. The smallest differences in the δ values are shown by the signals for C2/C6 of the substituents ($\Delta\delta \approx 0.6$). For the carbon atoms of the benzene ring and carbon atoms C3/C5 of the substituents, the difference is larger ($\Delta\delta \approx 2.7$ and 2.5, respectively).

The electrostatic activation of 4 is likewise illustrated by its hydrolysis. Whereas hexafluorobenzene is stable at normal pressure to aqueous sodium hydroxide solution,[18] 4 undergoes hydrolysis within 30 min in refluxing 0.1 N $NaHCO_3$ solution to give the corresponding pentakis-substituted phenolate 5 virtually quantitatively. The analogous introduction of a hydroxyl group under comparably mild conditions is also observed with hexacyanobenzene.[19] Apparently the almost purely electrostatic activation of this reaction through the set of cationic substituents in 4 is as effective as the −M activation of the cyano groups in hexacyanobenzene.

The reactions of 4 with reducing agents and nucleophiles are being investigated currently.

Experimental Procedure

4: To a solution of 1 (0.20 mL, 1.73 mmol) in anhydrous CH_3CN (30 mL) under N_2 was added 3 (2.50 mL, 13.8 mmol) and 2 (2.00 g, 16.4 mmol), and the solution was then stirred at reflux. After 7 d the resulting white precipitate was filtered off, washed first with CH_3CN (2 × 5 mL) and then CH_2Cl_2 (3 × 5 mL), and dried under high vacuum for 12 h. Yield: 2.71 g (92.1 %). The use of stoichiometric quantities of 2 and 3 afforded significantly lower yields, even after longer reaction times. Single crystals of 4 were obtained by boiling briefly in water followed by slow cooling. Correct C,H,N-analysis; ^1H NMR (399.65 MHz, CD_3NO_2/CF_3COOD, 20 °C, TMS): $\delta = 8.21$ (d, $^3J(H,H) = 8.0$ Hz, 12 H; H-2/H-6), 7.02 (d, $^3J(H,H) = 8.0$ Hz, 12 H; H-3/H-5), 3.31 (s, 36 H; CH_3); ^{13}C NMR (100.4 MHz, CD_3NO_2/CF_3COOD, 20 °C, TMS): $\delta = 157.79$ (s; C4, DMAP), 141.12 (s; C2/C6, DMAP), 140.99 (s; benzene), 121.48 (q, $^1J(C,F) = -317.0$ Hz; CF_3), 111.07 (s; C3/C5, DMAP), 41.66 (s; CH_3); ^{19}F NMR (470.4 MHz, D_2O/HCl, 20 °C, C_6F_6): $\delta = -79.6$ (s; CF_3); IR (KBr): $\tilde{\nu}$[cm^{-1}] = 3060w, 3050w, 1650s, 1600m, 1480w, 1440m, 1410w, 1350vw, 1330vw, 1260vs, 1230s, 1150s, 1060vw, 1030s, 810m, 790w, 750vw, 630s.

5: A suspension of 4 (1.00 g, 0.59 mmol) in aqueous $NaHCO_3$ solution (0.1 N, 30 mL) was heated for 30 min at reflux. The solution was then cooled to 5 °C and the resulting pale yellow precipitate filtered off, washed with cold water (1 × 5 mL) followed by CH_2Cl_2 (2 × 5 mL), and then dried under high vacuum for 12 h. Yield: 0.72 g (94.2 %). Correct C,H,N-analysis; ^1H NMR (400.05 MHz, CD_3CN, 20 °C, TMS): $\delta = 8.25$ (d, $^3J(H,H) = 7.57$ Hz, 2 H; H-2/H-6), 8.19 (d, $^3J(H,H) = 7.33$ Hz, 4 H; H-2/H-6), 8.03 (d, $^3J(H,H) = 7.57$ Hz, 4 H; H-2/H-6), 6.87 (d, $^3J(H,H) = 7.57$ Hz, 4 H; H-3/H-5), 6.78 (d, $^3J(H,H) = 7.33$ Hz, 4 H; H-3/H-5), 6.72 (d, $^3J(H,H) = 7.57$ Hz, 2 H; H-3/H-5), 3.17 s (12 H; CH_3), 3.13 s (12 H; CH_3), 3.12 s (6 H; CH_3); ^{13}C NMR (100.50 MHz, CD_3CN, 20 °C, TMS): $\delta = 164.56$ (s; C1; phenol), 157.51 (s; C4, DMAP), 157.34 (s; C4, DMAP), 157.25 (s; C4, *p*-DMAP), 144.23 (s; C2/C6, *p*-DMAP), 143.86 (s; C2/C6, DMAP), 141.96 (s; C2/C6, DMAP), 137.50 (s; C3/C5, phenol), 132.31 (s; C2/C6, phenol), 121.93 (q, $^1J(C,F) = -321.4$ Hz; CF_3), 113.70 (s; C4, phenol), 109.51 (s; C3/C5, DMAP), 109.48 (s; C3/C5, *p*-DMAP), 108.95 (s; C3/C5, DMAP), 41.09 (s; CH_3), 41.04 (s; *p*-CH_3), 40.87 (s; CH_3); IR (KBr): $\tilde{\nu}$[cm^{-1}] = 3050w, 1650vs, 1580m, 1540m, 1440w, 1405m, 1270s, 1220s, 1150s, 1030s, 820w, 750vw, 720vw, 630s.

Compound 5 may be protonated with trifluoromethanesulfonic acid in CH_2Cl_2 to give pentakis(4-dimethylamino-1-pyridinio)phenol pentakis(trifluoromethanesulfonate).

6: To a solution of 1,3,5-trifluorobenzene (0.30 mL, 2.90 mmol) in anhydrous chlorobenzene (20 mL) under N_2 was added 3 (2.00 mL, 11.1 mmol) and 2 (1.60 g, 13.1 mmol), and the solution was then stirred at reflux. After 9 d the resulting white precipitate was filtered off, washed with Et_2O (2 × 5 mL), and then dried under high vacuum for 12 h. Yield 1.06 g (41.1 %, unoptimized). Correct C,H,N-analysis; ^1H NMR (400.05 MHz, CD_3CN, 20 °C, TMS): $\delta = 8.44$ (d, $^3J(H,H) = 7.93$ Hz, 6 H; H-2/H-6), 8.01 (s, 3 H; C-H, benzene), 7.10 (d, $^3J(H,H) = 7.93$ Hz, 6 H; H-3/H-5), 3.30 (s, 18 H; CH_3); ^{13}C NMR (100.50 MHz, CD_3CN, 20 °C, TMS):

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δ = 157.93 (s: C4, DMAP), 144.74 (s: C1/C3/C5, benzene), 141.85 (s: C2/C6, DMAP), 122.05 (q, $^1J(C,F) = -319.5$ Hz; CF_3), 121.17 (s, C2/C4/C6, benzene), 109.18 (s: C3/C5, DMAP), 41.22 (s; CH_3); IR (KBr): $\tilde{\nu}$[cm^{-1}] = 3060w, 1650vs, 1640vs, 1605s, 1570s, 1530w, 1450m, 1400m, 1350w, 1260s, 1160s, 1090w, 1030s, 830m, 820m, 730w, 630s.

7: To a solution of pentafluorobenzene (0.10 mL, 0.91 mmol) in anhydrous CH_3CN (20 mL) under N_2 was added **3** (0.95 mL, 5.26 mmol) and **2** (0.800 g, 6.55 mmol), and the solution was then stirred at reflux. After 8 d the reaction mixture was concentrated to dryness and the resulting white solid suspended in CH_2Cl_2 (20 mL), filtered off, washed with CH_2Cl_2 (3 × 5 mL), and then dried under high vacuum for 12 h. Yield 0.808 g (62.1%, unoptimized). Correct C,H,N-analysis; ^{1}H NMR (400.05 MHz, CD_3CN, 20 °C, TMS): δ = 8.65 (s, 1H; benzene), 8.13 (d, ^3J(H,H) = 7.94 Hz, 4H; H-2/H-6), 8.07 (d, ^3J(H,H) = 8.67 Hz, 2H, H-2/H-6), 8.02 (d, ^3J(H,H) = 8.06 Hz, 4H; H-2/H-6), 7.01 (d, ^3J(H,H) = 7.94 Hz, 4H; H-3/H-5), 6.91 (d, ^3J(H,H) = 8.06 Hz, 4H; H-3/H-5), 6.88 (d, ^3J(H,H) = 8.67 Hz, 2H; H-3/H-5); 3.25 (s, 12H; CH_3), 3.21 (s, 12H; CH_3), 3.20 (s, 6H; CH_3); ^{13}C NMR (100.50 MHz, CD_3CN, 20 °C, TMS): δ = 158.00 (s; C4, DMAP), 157.67 (s; C4, DMAP), 157.63 (s; C4, DMAP), 142.40 (s; C1/C5, benzene), 141.43 (s; C2/C6, DMAP), 140.97 (s; C2/C6, DMAP), 138.31 (s; C3, benzene), 137.96 (s; C2/C4, benzene), 132.88 (s; C6, benzene), 121.83 (q, $^1J(C,F) = -319.8$ Hz; CF_3), 110.65 (s; C3/C5, DMAP), 110.55 (s; C3/C5, DMAP), 109.81 (s; C3/C5, DMAP), 41.44 (s; CH_3), 41.37 (s; CH_3); IR (KBr): $\tilde{\nu}$[cm^{-1}] = 3100s, 2980w, 1660s, 1580s, 1510s, 1450s, 1350m, 1270vs, 1170s, 1060m, 1030s, 940m, 830s, 790m, 750m, 740w, 730w, 710w, 630s.

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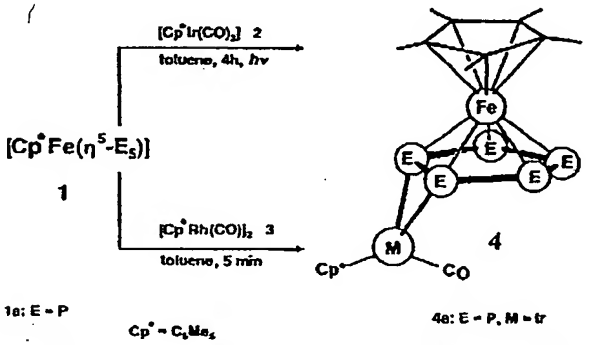
η^5:η^2 Coordination of a *cyclo*-E_5 Ligand, E = P, As**

Michaela Detzel, Gabriele Friedrich, Otto J. Scherer,* and Gotthelf Wolmershäuser

Dedicated to Professor Manfred Regitz on the occasion of his 60th birthday

In the extensive literature[1] on ferrocene and its derivatives there is to our knowledge no example in which in addition to η^5 coordination, the η^2-side-on coordination to one of the two parallel five-membered rings has been achieved. In lanthanoid complexes with bent sandwich structures, a (μ-η^5:η^2-C_5H_5) structural unit is found in $[(C_5H_5)_3La]_n$,[2a] a type of coordination which is also discussed in $[Cp^*_2Sm(\mu\text{-}C_5H_5)SmCp^*_2]$.[2b] We have now been able to show that the sandwich complexes **1** (**1a**: E = P;[3] **1b**: E = As[4]), which are iso(valence)electronic and isolobal to ferrocene $FeCp_2$, can be converted to the complexes **4** in which the unusual μ-η^5:η^2-*cyclo*-E_5 coordination (**4a**: E = P; **4b**: E = As) is realized. So far only terminal η^1 coordination of 16 VE complex fragments could be verified both in sandwich complexes with five-membered rings consisting of P(As) and CR building blocks[5a] as well as in **1**.[5b]



Compound **4a** forms brown crystals that can be manipulated in air for short periods, **4b** dark brown crystals that are air and moisture sensitive. They are sparingly soluble in pentane and dissolve well in toluene and dichloromethane. The five P atoms of the *cyclo*-P_5 ligand give rise in the ^{31}P NMR spectrum to an

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[+] X-ray crystal structure analyses.

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